Fetal germ cell differentiation and the impact of the somatic cells

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Declaration

The research described in this thesis is the sole work of the author, except where acknowledgement is made and is not submitted in support of another degree or qualification at the University of Edinburgh or any other educational institute.

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Abstract

Specification of a germ cell lineage and appropriate maturation are essential for the transfer of genetic information from one generation to the next. Germ cells form from pluripotent precursor cells that migrate into the gonadal ridge and undergo commitment to either the female or male lineage. In the fetal ovary, germ cells enter meiotic prophase I, then arrest at the diplotene stage; in the testis germ cells do not begin meiosis until puberty. Abnormal differentiation of germ cells can result in malignant transformation. Somatic cells play a key role in modulating the developmental fate of the germ cells. Research into germ cell development during fetal life has almost exclusively focused on studies in rodents, but we, and others, have reported several fundamental differences in the expression of germ cell specific markers in the human compared with the mouse. The studies described in this thesis have investigated germ cell-specific gene expression and the possible impact of the somatic cells during development. This was achieved by studying human fetal gonads obtained during the 1st and 2nd trimesters of pregnancy and through the use of both wild-type and mutant mouse ES cell lines.

Studies on germ cells in the human fetal testis have extended the findings of others, and confirmed that germ cell populations at different stages of maturation co-exist in the human fetal testis, a situation that is in contrast to that in rodents. For example expression of M2A and AP2γ was restricted to the OCT4-positive gonocyte population, while VASA and NANOS1 were localised exclusively to the to the OCT4-negative prespermatogonia. DAZL was expressed in both populations. Analysis also revealed that both the gonocyte and prespermatogonial populations proliferate throughout the 2nd trimester. Recent studies have implicated retinoic acid (RA) in the control of meiotic entry in germ cells of the fetal mouse ovary. In this study we demonstrated for the first time that two genes implicated in the action of RA in mouse gonad, *STRA8* and *NANOS2*, are also expressed in a similar sex-specific-manner in the human fetal gonads, and that the RA receptors are present in both somatic and germ cells suggesting that RA may regulate germ cell function in the human as well as the mouse. However, whilst the mesonephros appears to be the

primary site of RA synthesis in the mouse our initial studies indicate that in the human the gonad itself may be a more likely site of RA biosynthesis. In the fetal mouse testis, RA is degraded by the enzyme Cyp26b1 present in the somatic cells and germ cells do not enter meiosis, our novel findings suggest that *CYP26B1* is more abundant in the human fetal ovary than the testis, suggesting that meiotic entry may be controlled by an alternative signalling pathway in the human.

One of the methods that can aid our understanding of somatic cell gene expression in the gonad is in vitro culture. To date, there have been no published reports of the successful in vitro culture of somatic cells from the human fetal testis. In the current study, populations of human somatic cells were dissociated and maintained in vitro and characterised. Analysis demonstrated that cells expressing mRNAs characteristic of Sertoli cells, Leydig cells and peritubular myoid (PTM) cells were present initially, but long-term culture resulted in downregulation in expression of mRNAs specific for Sertoli cells and Leydig cells, suggesting that these cells either failed to survive or underwent alterations to their phenotype. In contrast PTM/fibroblast cells proliferated in vitro and initially maintained androgen receptor expression. These cultures therefore hold promise for studies into the signalling or cell-cell interactions in testicular somatic cells especially those relevant to the PTM population.

Several studies have claimed differentiation of putative germ cells from ES cells. In the current study, analysis of mouse ES cell lines has expanded on results showing that ES cells and early germ cells express a number of genes in common. Kit signalling was shown to be important for ES cell survival as they differentiate although expression of Kit was heterogeneous. We also demonstrated that ES cells that did not express Kit displayed a decreased expression of the early germ cell genes *Blimp1*, *Fragilis* and *Stella*, implicating Kit signalling in the control of germ cell-associated gene expression in ES cells. This may be important to future studies optimising germ cell derivation from ES cells.

In conclusion, this study has demonstrated important differences in protein expression patterns in germ cells of the human fetal testis compared to the mouse,

and has raised questions about whether the proposed mechanism controlling meiotic entry of germ cells in the mouse can be applied to the human. The establishment of a system for culturing human fetal gonadal somatic cells may lead to further understanding of gene expression and development in the human fetal testis, and data suggest that the Kit/Kitl signalling system may influence germ cell gene expression in mouse ES cells.

Presentations relating to this thesis

Expression of VASA in germ cells of the human fetal testis. Oral presentation at the 16th European Testis workshop, April, 2006, Bad Aibling, Germany.

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Abbreviations

Abbreviation	Definition
3βHSD	3-β-hydroxysteroid dehydrogenase
ACTR IIB	activin receptor IIB
ACTRIIA	activin receptor IIA
ALDH1A2	aldehyde dehydrogenase 1 family, member A2
ALDH1A3	aldehyde dehydrogenase 1 family, member A3
ALK2	cytochrome P450 alkane hydroxylase 2
ALK4	cytochrome P450 alkane hydroxylase 4
AMH	anti-Mullerian hormone
Ap2γ	activating enhancer binding protein 2 γ
AR	androgen receptor
Blimp1	B-lymphocyte induced maturation protein 1
CIS	carcinoma in situ
CYP26A1	cytochrome p45026A1
CYP26B1	cytochrome p45026B1
CYP26C1	cytochrome p45026A1
DAB	3,3'-diaminobenzidine
DES	desmin
DHH	desert hedgehog
e	embryonic day
ES	embryonic stem
GATA4	GATA-binding factor 4,
GFP	green fluorescent protein
hEG	human embryonic germ
Hoxa1	homeobox A1
HFT	human fetal testis

ICM	inner cell mass
Kit	proto-oncogene tyrosine-protein kinase receptor
Kitl	Kit ligand
LNA	locked nucleic acid
Mvh	mouse vasa homologue
OCT4	octamer-binding transcription factor 4
p	passage
PGC	primordial germ cell
PTM	peritubular myoid cell
RA	retinoic acid
RAR	retinoic acid receptor
RXR	retinoid X receptor
SCCp450	side chain cleavage p450
sem	standard error of the mean
SF1	steroidogenic factor 1
SMAD2	mothers against DPP homolog 2
SOX2	SRY-box2
SOX9	SRY-box 9
SRY	sex-determining region of the Y chromosome
STRA8	stimulated by retinoic acid gene 8
TBS	tris buffered saline solution
TGCT	testicular germ cell tumours
VIM	vimentin
WT	wild-type
WT1	Wilms tumour 1
α-SMA	α -smooth muscle actin

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1 Literature Review

1.1 General Introduction

During fetal development, the genital ridges appear as a thickening of the intermediate mesoderm (Wartenberg, 1981). Primordial germ cells (PGCs) arise from the proximal epiblast as a result of extraembryonic signals (Lawson and Hage, 1994) and migrate into the gonad by e10.5 in the mouse and five weeks gestation in the human, as dated from last menstrual period (Wartenberg, 1981). In the ovary nests of syncytial germ cells form that are linked by cytoplasmic bridges (McNatty et al., 2000), these cells enter meiosis and arrest at the diplotene stage of the first meiotic division (McLaren and Southee, 1997) at which time primordial follicles are formed (Hirshfield, 1992). In the male gonad, the germ cells become enclosed within the testicular cords and proliferate but do not enter meiosis until puberty. The somatic cells are thought to play an important role in germ cell maturation. Recent studies in the mouse suggest that retinoic acid (RA) induces germ cells in the female to enter meiosis, while the expression in the testis cords of the RA metabolising enzyme Cyp26b1 inhibits this in the male (Bowles and Koopman, 2007). The majority of studies on mammalian germ cell development have focused on the mouse. However, previous work in our laboratory has shown that in the second trimester human fetal testis, germ cells are morphologically distinct and can be categorised into OCT4-positive gonocytes and OCT4-negative prespermatogonia (Gaskell et al., 2004). This is in contrast to the situation in the rodent testis where germ cells are homogenous. The primary aim of this thesis was to further understand germ cell development and the influence of the somatic cells in the human fetal gonad. These studies have used human gonads obtained following elective termination of pregnancy during the 1st and 2nd trimester. This thesis also aimed to understand germ cell gene expression in mouse embryonic stem (ES) cells, using wild-type and mutant cells.

1.2 Development of gonadal somatic cells

1.2.1 Morphological development

As the gonad develops, it is initially 'bipotential', with the ability to form either ovaries or testes. The mammalian gonad forms within the urogenital ridge, where it appears as a thickening of the intermediate mesoderm, situated on the ventromedial side of the mesonephros at e10.5 in the mouse (McLaren, 1991a), and 4 weeks gestation in the human (Wartenberg, 1981) (Figure 1-1a). The gonad forms through proliferation of the coelomic epithelium within the medio-lateral side of the mesonephros (Wartenberg, 1981) (Figure 1-1b).

Sexual differentiation begins at e10.5 in the mouse and 6 weeks gestation in the human (McLaren, 1991b) (Wartenberg, 1981; Wartenberg, 1982). As the testis develops, cell proliferation increases within the coelomic epithelium between e11.3 and e12.1 in the mouse (Schmahl et al., 2000), and between 6-8 weeks gestation in the human (Wartenberg, 1981)(Figure 1-1c). In males, the somatic Sertoli cells are thought to arise from the coelomic epithelium (Karl and Capel, 1998). As they differentiate, they polarize and aggregate around the germ cells, which have migrated into the developing gonad by e11.5 in the mouse and week 5 of gestation in the human (Wartenberg, 1981) (refer to section 1.3.1).

Development of the testis is driven by the Sertoli cells, once the Sertoli cells have differentiated, other bipotential cell types of the gonad are committed to the male pathway (McLaren and Southee, 1997). The Sertoli cells themselves become surrounded by the peritubular myoid cells (PTM) cells, which form a single flat cell layer around the Sertoli cells allowing the male gonad to subsequently become organized into two distinct compartments: the testis cords, containing the germ cells and the Sertoli cells, and the interstitium which is located between the cords (Figure 1-2). Within the interstitium there are blood vessels, interstitial fibroblasts and Leydig cells. The Leydig cells form clusters of cells, often situated in close proximity to blood vessels (O'Shaughnessy et al., 2006) (Figure 1-2). The Leydig

cells are responsible for the production of androgens (Habert and Picon, 1984). For normal testis development to occur, cells must migrate into the male gonad from the adjoining mesonephros. Experimental studies have determined that mesonephric cells can develop into vascular endothelial cells, interstitial fibroblast and possibly PTM cells and Leydig cells (Buehr et al., 1993; Merchant-Larios and Moreno-Mendoza, 1998; Merchant-Larios et al., 1993; Nishino et al., 2001).

Migrating endothelial cells associate to establish the male-specific coelomic vessel (Figure 1-2) which increases blood flow through the developing testis, and is thought to be important for the efficient export of androgens (Brennan et al., 2002).

In the developing female gonad, the surface epithelium continues to proliferate, forming the cortical sex cords (Figure 1-1e). The germ cells are found closer to the surface of the gonad than the male and the cortical sex cords break up, and form clusters, each surrounding a single germ cell (Figure 1-1f). The cortical sex cords will differentiate into the granulosa cells, while the surrounding mesenchymal cells differentiate into the thecal cells (Konishi et al., 1986; Gilbert, 2003; McLaren, 1991a). The thecal cells and granulosa cells will together form the follicles.

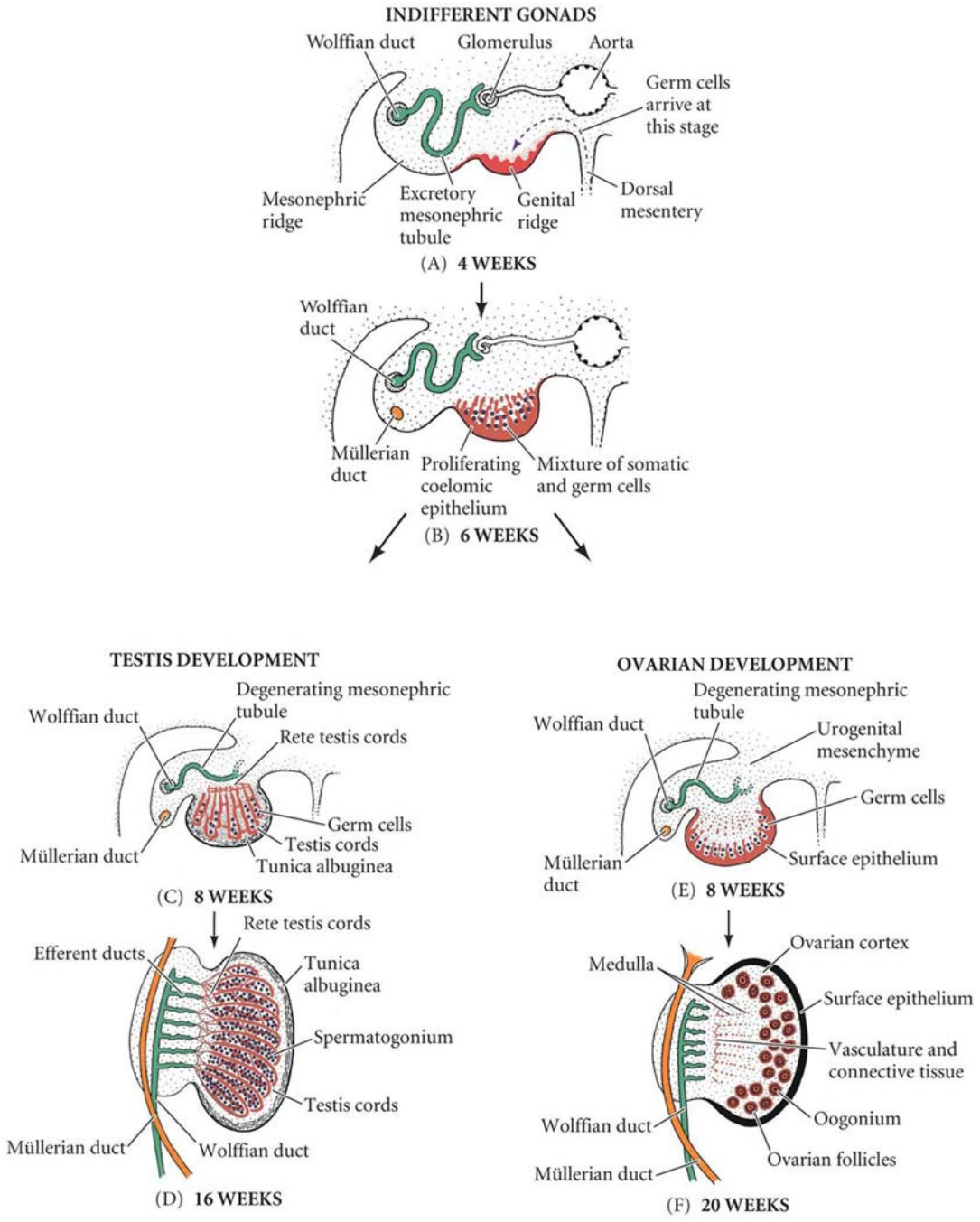

Figure 1-1 Summary of the differentiation of the human gonads shown in transverse section from Gilbert, 2003

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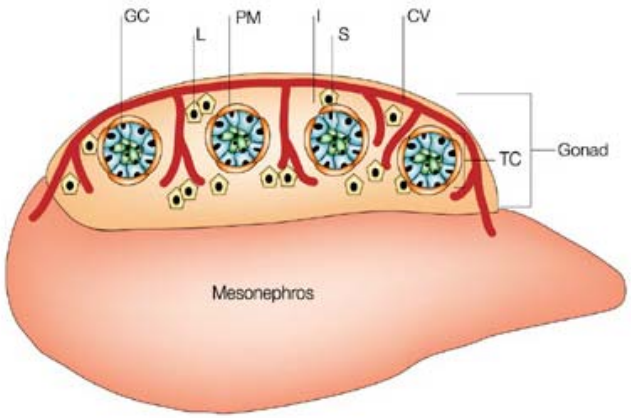

Figure 1-2 Structural organisation of the fetal testis at e12.5, from Brennan and Capel, 2004 (GC=germ cell, S=Sertoli cells, L=Leydig cells, PM=peritubular myoid cells, I=interstitial cells, CV=coelomic vessel, TC= testicular cords)

1.2.2 Molecules controlling Sertoli cell differentiation

1.2.2.1 SRY

In chimeric mice formed from XX and XY embryos, Sertoli cells were found to be over 90% genetically male, suggesting that Sertoli cell differentiation is dependent upon the presence of the Y chromosome (Palmer and Burgoyne, 1991). The gene responsible for sex determination is known as the sex determining region on the Y chromosome (*Sry*). *Sry* is the founding member of the Sox (Sry-related high mobility group HMG box) family of transcription factors. The majority of mammals determine their sex through *Sry* (Wallis et al., 2008). Mice which are genetically male but do not express *Sry* develop an ovary and appear phenotypically female (Gubbay et al., 1992; Lovell-Badge and Robertson, 1990), while XX mice with a transgenic autosomal copy of *Sry* develop a testis containing Sertoli cells and Leydig cells, and display male secondary sex characteristics (Koopman et al., 1990). In humans, *SRY* mutations have been shown to be responsible for 10% of sex reversal cases and often result in gonadal dysgenesis (Berta et al., 1990). Almost all mutations in *SRY* resulting in sex reversal are located within the highly conserved

HMG box, suggesting only minor functionality for other regions of the protein (Wilhelm et al., 2007). It is speculated that *Sry* acts through binding and bending of target DNA, allowing upstream and downstream factors to interact and thus enabling transcription (Harley et al., 1992; Giese et al., 1992; Jager et al., 1992; Harley et al., 2003).

In the mouse, Sry mRNA and protein are first detectable in the male gonad at e10.5, in a small number of somatic cells thought to be the pre-Sertoli cells (Albrecht and Eicher, 2001; Hacker et al., 1995). Sry is expressed in a transient manner, where it is initially restricted to the centre of the gonads, but then expands to the poles, before being quickly downregulated, first at the anterior pole and finally at the posterior pole, with protein expression completely lost by e12.5 (Hacker et al., 1995; Swain and Lovell-Badge, 1999). The timings of *Sry* are crucial for its functions, if expression begins too late, ovotestes are formed (Bullejos and Koopman, 2005), while a threshold level of *Sry* must be achieved for it to function efficiently (Harley et al., 2003). In the human *SRY* expression begins in the gonadal ridge at 44 days post-ovulation, once the cords form. Curiously, human *SRY* does not display the transient expression which occurs in the mouse, instead, expression is maintained throughout development (Hanley et al., 2000), and can even be detected in the adult testis (Salas-Cortes et al., 1999).

1.2.2.2 SOX9

Although the transcriptional targets of Sry have not been fully identified, one probable action of Sry is the upregulation of one of its family members, the Sry-like HMG-box protein 9 (*Sox9*) (Bowles et al., 2000) (Figure 1-3). The promoter of *Sox9* contains potential Sry-binding sites, although no direct molecular interaction between the two has yet been demonstrated (Bishop et al., 2000; Vidal et al., 2001). Sox9 is initially expressed throughout the genital ridge of both the male and female, however after the onset of expression of *Sry* in the male genital ridge, *Sox9* is dramatically upregulated by e11.5 (Kanai et al., 2005). Experiments with mice

expressing a Myc-epitope tagged Sry protein have shown that Sox9 upregulation is exclusive to those cells expressing Sry (Sekido et al., 2004). It is still to be established whether the only action of Sry is to regulate expression of *Sox9* or if it has other downstream targets. However, ectopic activation of *Sox9* in XX embryos resulted in female-to-male sex reversal, suggesting that *Sox9* may be the only important gene under Sry regulation (Bishop et al., 2000; Vidal et al., 2001). In the mouse, once Sox9 has reached a threshold level, Sry expression becomes repressed, possibly due to a Sox9-dependent negative feedback loop (Chaboissier et al., 2004; Sekido et al., 2004) and expression of *Sox9* is maintained in the testis thereafter (Morais da Silva et al., 1996). In humans, mutations in *SOX9* result in campomelic dysplasia, where patients have abnormalities consistent with a role for SOX9 in chondrogenesis (Wright et al., 1995), and the majority of male patients also display sex reversal (Foster et al., 1994; Wagner et al., 1994). In the human male gonad, *SOX9* expression follows a similar pattern to that of *SRY* (Hanley et al., 2000). Interestingly, *SOX9* transcripts have also been detected in the human fetal ovary after sex determination has occurred, despite being absent in the mouse ovary (Hanley et al., 2000).

Although the evidence suggests that *Sox9* is under the control of Sry, experiments have also shown that expression of *Sox9* can become activated in the gonad by factors other than *Sry*. In female mouse gonads lacking both oestrogen receptors, ERα and ERβ, sex reversal occurs postnatally, with granulosa cells upregulating Sox9 and apparently undergoing transdifferentiation into Sertoli cells (Couse and Korach, 1999; Dupont et al., 2003).

In addition to Sry, a number of other molecules have also been implicated in *Sox9* transcriptional control. For example, Steroidogenic factor 1 (Sf1) is an orphan nuclear receptor which is involved in the transcriptional regulation of genes encoding a number of steroidogenic enzymes in both the gonad and adrenal gland (Lala et al., 1992; Luo et al., 1994; Parker and Schimmer, 1997). Recent evidence suggests that both Sf1 and Sry bind to multiple elements within a gonad-specific enhancer on the

Sox9 gene. It appears that Sry and Sf1 can act together to upregulate *Sox9,* and in the mouse after Sry expression is lost, Sf1 and Sox9 cooperatively bind to the *Sox9* enhancer, allowing Sox9 expression to be maintained after Sry expression is lost, implicating Sox9 in the autoregulation of its own expression (Sekido and Lovell-Badge, 2008).

In the absence of Sry, Wilms Tumour 1 (WT1) may also act to control *Sox9* transcription. Wt1 is a zinc finger containing DNA binding protein which exists in multiple splice isoforms and can enhance or repress transcription (Hohenstein and Hastie, 2006; Yang et al., 2007). When *Wt1* is knocked-out specifically in Sertoli cells, although Sox9 is initially expressed, it becomes downregulated after Sry switches off, suggesting that in the absence of Wt1, Sox9 cannot be maintained, after Sry is downregulated (Gao et al., 2006).

Also implicated in the control of *Sox9* transcription is Gata4, a member of the Gata family of zinc finger proteins (Arceci et al., 1993; Tevosian et al., 2002; Viger et al., 2008). Gata4 is expressed in all somatic cells of the mouse testis, and is reported to be expressed at high levels in Sertoli cells by e11.5 (Viger et al., 1998). In mice, a mutation in the co-factor for Gata4, the so called Friend of Gata 2 (*Fog2*) (Tevosian et al., 2002) or a *Gata4* knock-in mutation (Crispino et al., 2001), which inhibits the interaction of Gata4 and Fog2, both result in identical phenotypes, with impaired cell proliferation, and defective Sertoli cell and Leydig cell development. In these two mutants, Sox9 levels are undetectable. Recently a Gata4/Fog2 transcriptional complex has been shown to be required for the transcription of *Sox9* (Manuylov et al., 2007).

In the mouse, twelve hours after the upregulation of *Sox9*, another of the Sox gene family members, *Sox8* is also expressed specifically in the Sertoli cells. Sox8 has been shown to perform a number of similar molecular roles to Sox9 (Schepers et al., 2003) but studies now suggest that it is functionally redundant (Chaboissier et al., 2004).

The cellular localisation of Sox9 appears to have important implications for its function. Initially Sox9 is detected in the cytoplasm of the undifferentiated gonad, but upon expression of *Sry*, the protein is phosphorylated and translocates to the nuclear compartment. The active transport of Sox9 across the nuclear membrane appears to be mediated by nuclear localisation signals contained within Sox9, for example, the C-terminal of Sox9 has been shown to bind importin β (Sudbeck and Scherer, 1997). When bound with importin β, Sox9 can interact with Ran-binding protein 2 (RanBP2), which mediates the nuclear translocation of importin β bound proteins (Stewart, 2007). Additionally, *in vitro* studies have shown that the N-terminal nuclear localisation signal of Sox9 is capable of binding calmodulin (Harley et al., 1996; Argentaro et al., 2003), which acts to facilitate the transportation of target proteins through the nuclear pore complex (Sweitzer and Hanover, 1996). Some *Sox9* XY sex reversal mutations display reduced binding to calmodulin, suggesting that this function may be necessary for sex determination (Sim et al., 2005).

In the female, Sox9 remains cytoplasmic, and expression is rapidly downregulated (Morais da Silva et al., 1996). Studies in the mouse suggest that in the female, the intracellular location of Sox9 is also important. For example, the Sox9 HMG box contains a nuclear export signal, which allows it to exit the nucleus, and inhibition of this in cultured XX mouse gonads results in female-to-male sex reversal (Gasca et al., 2002).

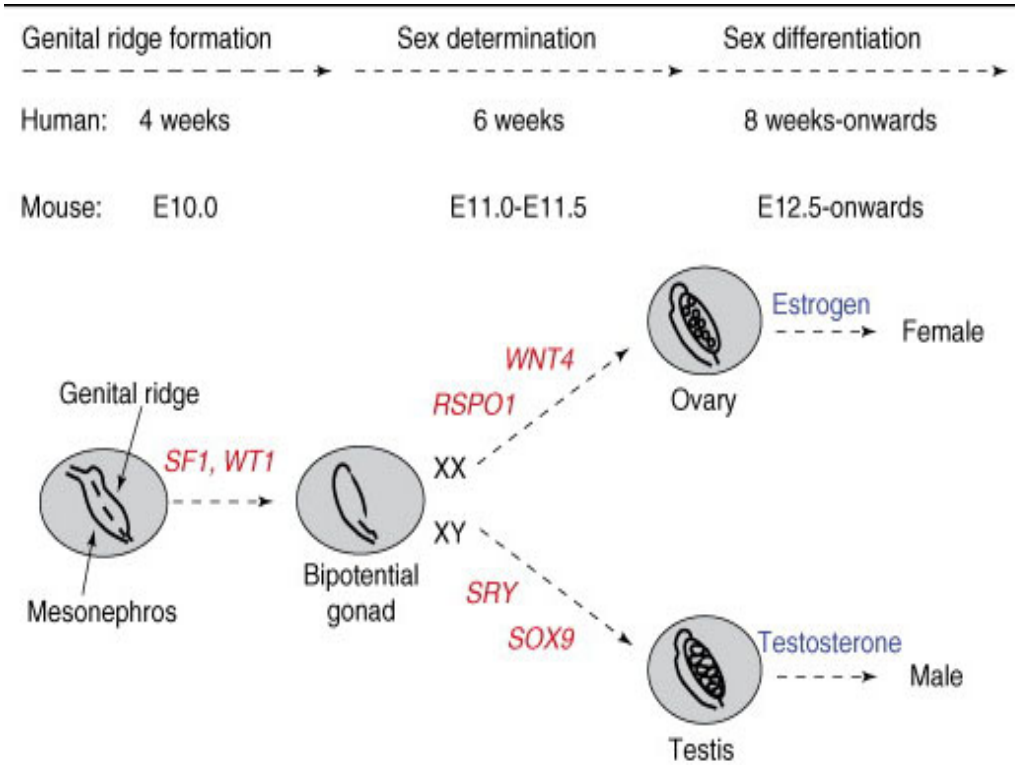

Figure 1-3 Summary of the molecular control of gonadal development. From Sim et al., 2008

1.2.2.3 Paracrine signalling mechanisms controlling Sertoli cell development

Studies have shown that in an XX/XY chimeric mouse, 90% of the cells which form Sertoli cells are XY, consistent with evidence that commitment to the Sertoli cell lineage is critically dependent on expression of *Sry* (Palmer and Burgoyne, 1991; Wilhelm et al., 2005). However, as approximately 10% of the Sertoli cells in the chimeric mouse are XX, this suggests that a possible paracrine signalling system may be actively recruiting XX cells or cells that have not upregulated *Sry* into the Sertoli cell lineage. Candidate molecules involved in paracrine signalling at the time of sex determination are discussed below.

1.2.2.3.1 Prostaglandin D2

Prostaglandin D2 (PGD2) is a paracrine signalling molecule that is produced by the enzyme prostaglandin D synthase (Urade and Hayaishi, 2000). Studies suggest that PGD2 may act by inducing Sox9 nuclear translocation and subsequent Sertoli cell differentiation (Figure 1-4). Expression of prostaglandin D synthase was found to be highly upregulated in the male gonad at the time when sex determination is occurring, from e11.5 in both the Sertoli cells and the germ cells (Adams and McLaren, 2002; Malki et al., 2005). When XX gonads *in vitro* were treated with PGD2, they underwent masculinisation, with increased expression of *Sox9* and *Amh*, and evidence of PTM-like and Sertoli-like cells present within disorganised cord-like structures (Adams and McLaren, 2002; Wilhelm et al., 2005). Specificity was demonstrated by using an antagonist to the PGD2 receptor, prostaglandin DP receptor (Wilhelm et al., 2005) and *in vitro* PGD2 treatment induced Sox9 nuclear translocation (Malki et al., 2005). PGD2 may therefore have a role *in vivo* during testis development to recruit supporting cells into the male lineage (Malki et al., 2005). A paired Sox9/Sry binding site has been identified on the prostaglandin D synthase promoter, and there is functional evidence that Sox9, transactivates this promoter (Wilhelm et al., 2007). Mice lacking prostaglandin D synthase or the prostaglandin DP receptor display a normal gonadal phenotype, however this could reflect a degree of redundancy, as other prostaglandin synthases and receptors are expressed within the gonad (Adams and McLaren, 2002).

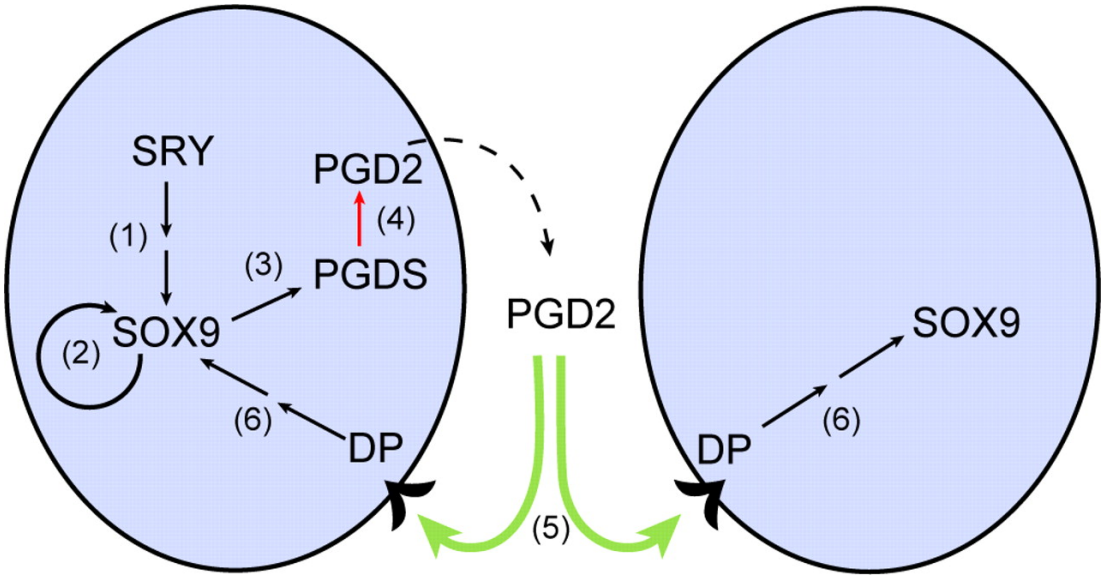

Figure 1-4. Summary diagram showing the role that PGD2 could have in the control of Sox9 in the mouse testis, from Wilhelm et al., 2007. 1) SRY induces the expression of Sox9. 2) Sox9 maintains its own expression in an auto regulatory loop 3) Sox9 upregulates prostaglandin D2 synthase (PGDS) 4) This leads to the expression of prostaglandin D2 and its secretion 5) Prostaglandin D2 binds to the DP receptor 6) This acts to upregulate Sox9 in either a paracrine or autocrine manner.

1.2.2.3.2 Fibroblast growth factor 9 (FGF9)

Fibroblast growth factor 9 (FGF9), is a member of a large family of growth factors, important for a number of developmental processes (Ornitz and Itoh, 2001). A role for *Fgf9* in sex determination was highlighted after *Fgf9* homozygous null mice were found to display a range of sex reversal phenotypes, ranging from abnormal testis development to complete sex reversal (Colvin et al., 2001). Fgf9 can be detected at e11.5 in gonads of both sexes, but later becomes restricted to the testis cords of the XY gonad (Schmahl et al., 2004). *Fgf9* may therefore play a role in Sertoli cell induction, proliferation and differentiation and may stimulate the expression of *Sox9*. In *Fgf9* mutant mice, *Sry* and *Sox9* are initially expressed as normal, but expression of *Sox9* is not maintained, and there is increased expression of female specific genes. Further evidence comes from *in vitro* studies where Fgf9

induces *Sox9* expression in XX somatic cells (DiNapoli et al., 2006; Kim et al., 2006), and similar results have been found in cell lines (Schaefer et al., 2003).

The actions of Fgf9 may be mediated by binding to the Fgf receptor 2 (Fgfr2), which is highly expressed in a male-specific manner, on the membrane of actively proliferating coelomic epithelial cells and the nucleus of pre-Sertoli cells (Schmahl et al., 2004). Mouse conditional knock-outs of *Fgf2r* phenocopy the *Fgf9* mutants (Kim et al., 2007; Bagheri-Fam et al., 2008).

1.2.2.3.3 FGF9 and WNT4 functional antagonism

In female mice with disrupted *Wnt4* signalling, expression of 'testis-specific' genes such as *Fgf9, Sox9* and *Dhh* is upregulated compared with controls, implicating Wnt4 in the repression of the male pathway (Jeays-Ward et al., 2004). *Wnt4* is expressed at e11 in the indifferent gonad, but by e11.5 it becomes downregulated in the male genital ridge, although expression is maintained in the mesonephroi of both sexes (Vainio et al., 1999). Both Wnt4 and the glycoprotein follistatin appear to be required for early ovarian development. Wnt4 appears to be an upstream regulator of follistatin (Yao et al., 2004) and both appear to inhibit endothelial cell migration and coelomic vessel formation, essential processes for successful testis development (Jordan et al., 2003). Wnt4 may act in conjunction with R-spondin1 to antagonize the male pathway (Figure 1-3). R-Spondin1 is a secreted protein implicated in the Wnt pathway (Blaydon et al., 2006) and mutations in the R-Spondin1 gene have been identified in XX patients who develop testes (Parma et al., 2006). Both Wnt4 and R-Spondin1 have been shown to activate the canonical Wnt pathway (Kim et al., 2006) resulting in stabilization of β -catenin, that acts as a cofactor for transcriptional activation. A recent study has shown that ectopic expression of β -catenin in somatic cells of the XY gonad can block the male pathway and result in XY sex reversal (Maatouk et al., 2008).

The addition of Fgf9 to XX gonads results in a downregulation of Wnt4, implicating Fgf9 in the inhibition of Wnt4 signalling, while in *Wnt4* mutants *Fgf9* levels are elevated (Kim et al., 2006). So Wnt4 and Fgf9 may antagonistically control the fate of the bipotential gonad. It is hypothesised that Sry may influence the Wnt4/Fgf9 balance, by upregulating the expression of Sox9. Sox9 may then act to upregulate *Fgf9*, and Fgf9 then in turn maintains the expression of *Sox9*, and downregulates *Wnt4* (Kim and Capel, 2006) (Figure 1-5).

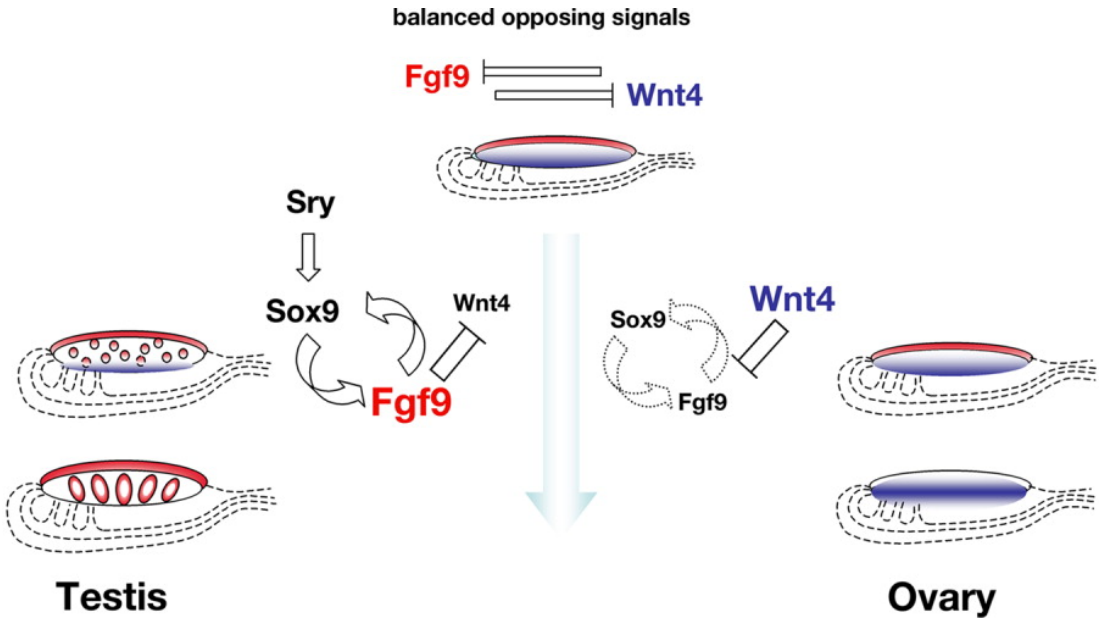

Figure 1-5 Molecular antagonism possibly controlling the fate of the developing testis, from Kim and Capel, 2006

1.2.2.4 Anti-müllerian Hormone (AMH)

Anti-müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a glycoprotein that shares homology with the transforming growth factor β (TGFβ) superfamily. AMH has been localised to the endoplasmic reticulum and Golgi apparatus of fetal and neonatal Sertoli cells (Tran and Josso, 1982; Hayashi et al., 1984). This protein was first identified because of the critical role it plays in the regression of the Müllerian duct (Jost et al., 1953; Josso et al, 2002; Josso et al., 2001; Josso, 2008). The protein is also expressed in the granulosa cells of the

mature follicle (Rajpert-De Meyts et al., 1999), where it is a useful marker in the follicular fluid for polycystic ovary syndrome (Cook et al., 2002).

In the mouse testis, Amh expression begins around 20 hours after the onset of *Sry* expression (Hacker et al., 1995). In mouse Sertoli cells Amh expression increases until e19.5, and then decreases significantly post-partum (Rey et al., 1993; Schwindt et al., 1997). In the human, AMH is expressed by 8 weeks gestation (de Santa Barbara et al., 2000; Rajpert-De Meyts et al., 1999) and its expression declines during puberty, at the onset of meiosis, coincident with the time when there is a surge in androgens (Gaskell et al., 2004; Hirobe et al., 1992). The mechanisms for this decline are still not understood as expression of AMH still decreases in the absence of meiotic germ cells and no direct role for androgens has been demonstrated (Rajpert-De Meyts et al., 1999).

AMH acts upon its target cells by binding to its type II receptor present on the cell membrane, and then recruiting the type I receptor and activating downstream Smads (Rajpert-De Meyts et al., 1999; Rey et al., 2003)(refer to Figure 1-17 for a summary of TGFβ signalling). The type II receptor has been localised to the mesenchymal cells situated adjacent to the Müllerian duct epithelium. It has also been found to be expressed in the fetal testis, and postnatal Sertoli cells (di Clemente et al., 2003; Josso et al., 2001; Baarends et al., 1995).

A number of genes controlling the transcription of *AMH* have now been identified (Figure 1-6), and these appear to be conserved (Baarends et al., 1994; Lasala et al., 2004; Tremblay and Viger, 1999). For example, SOX9 has been shown to be required to bind to its specific response element within the AMH promoter, prior to AMH transcription (Arango et al., 1999), while AMH is switched on shortly after SOX9 translocates into the nucleus in both the mouse (Morais da Silva et al., 1996) and human (de Santa Barbara et al., 2000).

SF1 may also have an important role in controlling the expression of *AMH*. Within the *AMH* promoter, two SF1 binding sites have been identified (Shen et al., 1994) (Arango et al., 1999), and SF1 has been shown to directly regulate *AMH* expression *in vitro* and *in vivo* (Giuili et al., 1997). A potential role for GATA4/FOG2 binding within the *AMH* promoter has also been found. The *AMH* promoter contains 2 GATA4 binding sites and GATA4 has been shown to enhance *AMH* promoter activity by directly binding with the DNA (Viger et al., 1998). Furthermore, one of the GATA4 binding sites on the *AMH* promoter lies adjacent to an SF1 binding site (Tremblay and Viger, 1999). Through the use of reporter assays, GATA4 and SF1 were found to be capable of direct protein-protein interaction, mediated by the zinc finger region of GATA4, and when co-expressed, both GATA4 and SF1 can activate the *AMH* promoter (Tremblay and Viger, 1999) (Figure 1-6).

Within the *AMH* promoter, WT1 has been shown to bind to a GC-rich sequence, possibly initiating transcription (Hossain and Saunders, 2003) (Figure 1-6 a). WT1 may in fact act by synergizing with SF1 and SOX9 to enhance *AMH* transcription (Hossain and Saunders, 2003). A recent study has also shown that the regulation of the *AMH* promoter by WT1 is also enhanced by WT1/GATA4 synergism (Miyamoto et al., 2008).

In contrast, the DAX1 protein may act to repress *AMH* transcription. *DAX1* encodes a member of the nuclear hormone receptor family and is thought to act as a transcriptional silencer (Muscatelli et al., 1994; Zanaria et al., 1994). DAX1 may repress *AMH* expression by disrupting GATA4/SF1 and WT1/SF1 synergisms by competing for direct interaction with SF1 (Nachtigal et al., 1998; Tremblay et al., 2001a) (Figure 1-6b).

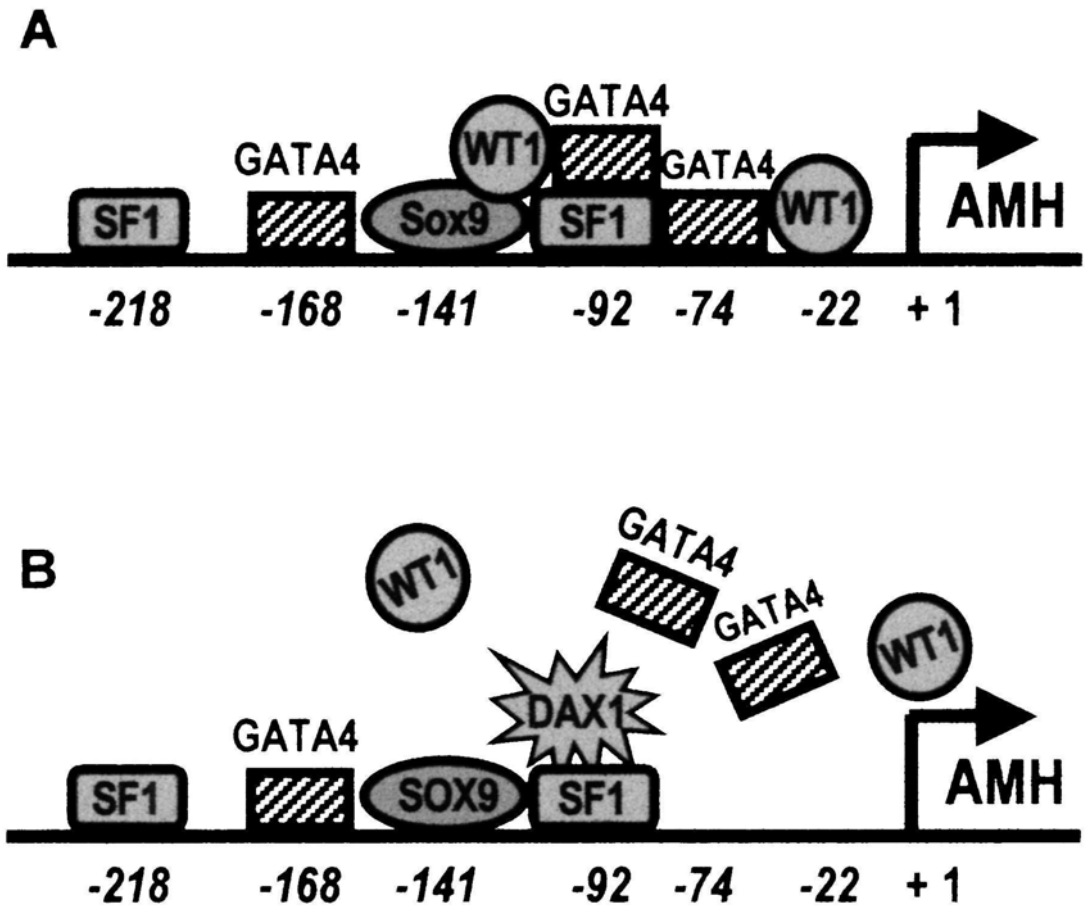

Figure 1-6. Molecular control of AMH expression in the human. SOX9 binds to a specific response element within the AMH promoter, SF1, GATA4 and WT1 also bind to the AMH promoter. GATA4/WT1 may also interact with WT1. b) DAX1 may also interact with SF1 and disrupt GATA4/SF1 and WT1/SF1 synergism, from Lasala et al., 2004.

1.2.3 Mesonephric cell migration

A key process specific to the developing testis is the migration of cells from the mesonephros (Buehr et al., 1993;Merchant-Larios et al., 1993). Migration occurs between e11.5-e16.5 in the male mouse (Martineau et al., 1997) and although, due to the lack of specific markers for certain cell types, it is difficult to ascertain the exact identity of cells that do migrate, it is speculated that endothelial, PTM and perivascular cells all migrate to the developing gonad (Merchant-Larios et al., 1993 Martineau et al., 1997; Nishino et al., 2001). Additionally, some cells that have

migrated into the gonad have been shown to give rise to Leydig cells (Merchant-Larios and Moreno-Mendoza, 1998;Nishino et al., 2001). Migration of mesonephric cells appears to be essential for development of the testis cords (Tilmann and Capel, 1999) as studies have shown that if the mesonephros is cultured separately from the gonad or if a physical barrier is placed between the two, migration does not occur and the cords do not form (Buehr et al., 1993; Tilmann and Capel, 1999).

What controls the migration of these cells, and the factors which make it specific to the male are not fully understood. *Sry* has been shown to be required for the migration of cells from the mesonephros (Capel et al., 1999). Furthermore, the culture of an XY gonad on the surface of an XX gonad resulted in the migration of cells from the mesonephros into the XX gonad (Tilmann and Capel, 1999), suggesting that a secreted molecule from the male gonad attracts these cells. Studies using organ culture have shown that a number of growth factors can induce mesonephric migration. For example, the addition of recombinant Amh to cultures containing XX gonads, induced mesonephric cell migration and the formation of the testis cords (Behringer et al., 1990; Bezard et al., 1987). But since *Amh* deficient XY mice, display no abnormalities in testis cord formation, it appears that there is a degree of redundancy in the actions of *Amh* in the developing testis (Ross et al., 2003). Mesonephric cell migration can also be stimulated when gonadal explants are cultured in the presence of Fgf9 , and *Fgf9* -/- mice display defective mesonephric cell migration into the XY gonad, although this mutant phenotype can at least partly be attributed to the other testis defects resulting from a lack of Fgf9 (Colvin et al., 2001).

The addition of nerve growth factor (NGF) has also been claimed to stimulate mesonephric migration *in vitro* (Cupp et al., 2000; Cupp et al., 2003). Although when two of the NGF receptors *TrkA* and *TrkC*, are knocked-out, there is only minor disruption to the cords and slightly defective interstitial development (Cupp et al., 2002). *Pdgfrα* knock-out mice also display defects in mesonephric cell migration (Brennan et al., 2003), although this receptor is required in cells of the gonad, not the

mesonephros, suggesting that the cells of the gonad may be stimulated by Pdgf to produce an unknown factor which then acts to stimulate migration.

1.2.4 Leydig cell development

The Leydig cells develop as distinct fetal and adult lineages. The fetal Leydig cells are present from e12.5 in the mouse and numbers quickly decline after birth (Byskov, 1986; Habert et al., 2001), while in the human they are evident from 9 weeks gestation (Codesal et al., 1990; Ostrer et al., 2007). The origin of Leydig cells has not been fully established but they are believed to form from the differentiation of mesenchymal-like stem cells (Byskov, 1986; Ge et al., 2006). These stem cells may be derived from the coelomic epithelium (Karl and Capel, 1998) although a more recent study suggests that only a small number may arise from here (Brennan et al., 2003). They also may arise from migrating mesonephric cells, but following removal of the mesonephros at e11.5, a normal Leydig cell population is still formed, suggesting that these cells do not migrate or in the absence of migration, redundant cells can compensate (Buehr et al., 1993; Merchant-Larios and Moreno-Mendoza, 1998; Merchant-Larios et al., 1993).

Notably, the Sertoli cells appear to influence the development of Leydig cells, through paracrine signalling mechanisms. One factor thought to be involved is the oncogene, Pdgfα, which is secreted by the Sertoli cells, while the Pdgfα receptor, Pdgfrα is expressed within the interstitium. Mouse mutants for *Pdgfrα* display severe defects in Leydig cell development (Brennan et al., 2003), suggesting Pdgfα may play an important role in promoting the proliferation and/or differentiation of Leydig cells. Desert hedgehog (Dhh) is another secreted molecule, expressed by the Sertoli cells and the Dhh receptor Patched (Ptch1), is expressed by cells within the interstitium (Bitgood et al., 1996). Dhh is expressed at e11.5 in the Sertoli cells, while it is absent from the ovary (Clark et al., 2000). *Dhh* knock-out mice display poor partitioning of the interstitium and the cord regions, and impaired Leydig cell development (Yao et al., 2002) and Dhh is also claimed to play a role in upregulating

steroidogenic genes within the developing Leydig cells (Yao et al., 2002). Human males with mutations in *DHH* display fertility problems (Canto et al., 2005; Bitgood et al., 1996) and interestingly the levels of expression of DHH was found to be reduced in the testes of fetuses from smoking mothers (Fowler et al., 2008).

The primary function of the Leydig cells is the secretion of androgens that are essential for masculinisation of the external and internal genitalia (Clark et al., 2000; Hu et al., 2002; Welsh et al., 2008). In the Leydig cells steroid biosynthesis begins with the conversion of cholesterol to pregnenolone by the enzyme side-chain cleavage p450 (SCCp450) (or Cyp11a1). The enzyme 3β-dehydrogenase (3βHSD) then converts pregnenolone to progesterone. Progesterone is converted to androstenedione by cytochrome P450 17α-hydroxylase, and androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase (O'Shaughnessy et al., 2006) (Figure 1-7). Both Side Chain Cleavage p450 (SCCP450) and 3-β-hydroxysteroid dehydrogenase (3βHSD**)** are present throughout the 2nd trimester in the human (Murray et al., 2000; Gaskell et al., 2004). In the mouse, fetal androgen production begins at e13, while in the human it peaks between 13-14 weeks gestation (Tapanainen et al., 1981). There are substantial differences in the steroidogenic enzymes between the rodent and the human. For example, there are multiple, different human and rodent isoforms of 17β-hydroxysteroid dehydrogenase (Labrie et al., 1997), and these display differences in both function and substrate specificity. In the rodent, 4-6 functional 3βHSD genes and isoenzymes have been identified (Payne et al., 1997), while in the human there are only two (Rheaume et al., 1992).

The Leydig cells are also responsible for the production of insulin-like3 (Insl3), which is required for the first phase of testicular descent (transabdominal) (Ivell and Hartung, 2003; Klonisch et al., 2004).

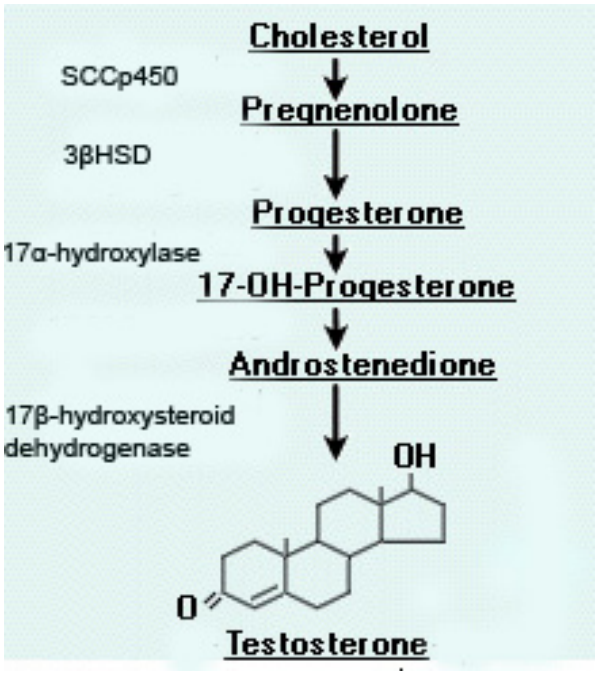

Figure 1-7 Summary of steroid biosynthesis in Leydig cell. Modified from Ogle, 1996

1.3 Germ cells

Germ cells have the important role of carrying the genome from one generation to the next, and enhancing the genetic diversity of the species. In order to do this, mammalian germ cells have to be specified within the gastrulating embryo and migrate into the gonad, while undergoing several rounds of cell division. Commitment to either the female or male lineage then occurs depending on their somatic cell environment. The germ cells must not only undergo development and maturation, but also successfully enter and complete meiosis at the appropriate time in males and females.

1.3.1 Primordial germ cells (PGCs)

1.3.1.1 PGC specification

In certain species such as Caenorhabditis elegans and Drosophila melanogaster, maternal factors from the germ plasm, deposited in the fertilised egg, are essential for

germ cell specification (Eddy, 1975). In mammals, there is no evidence of a germplasm, and the germ line is induced to form independently of maternal factors (McLaren and Lawson, 2005). Due to the difficulties in obtaining early human embryos, the early stages of germ cell specification have never been studied in the human. However germ cells at this stage of development has been comprehensively studied in the mouse. Although a number of molecular pathways are likely to be conserved and can be extrapolated to the human, it must be born in mind that the human embryo is morphologically and structurally distinct from the mouse at the time when germ cells are established and therefore humans may have evolved additional mechanisms to ensure specification of the germ cell lineage, which have yet to be elucidated (Clark and Reijo Pera, 2006).

As gastrulation occurs, mouse embryos consist of three tissue types: the epiblast, the extraembryonic ectoderm and the visceral endoderm. The extraembryonic ectoderm and the visceral endoderm give rise to the placenta and other extraembryonic tissues. The epiblast cells originate from the inner cell mass, and differentiate into all cell types of the embryo, including germ cells (Gilbert, 2003). The PGC population arises from the proximal epiblast, which lies adjacent to the extraembryonic ectoderm at the base of the allantois (Ginsburg et al., 1990; Lawson and Hage, 1994). Before gastrulation has occurred, PGCs have been shown not to be lineage restricted, as cells of the proximal epiblast will not form PGCs when transplanted to distal regions, while cells originating from the distal part of the epiblast transplanted proximally, are capable of forming PGCs (Tam and Zhou, 1996).

1.3.1.2 Signals controlling the induction of PGCs

The induction of cells into the germ cell lineage is at least partly controlled by signals from the extraembryonic ectoderm and visceral endoderm. Lineage studies by Lawson et al (1994) demonstrated that it is the regions of the proximal epiblast which sit within 1-2 cell diameters of the extraembryonic ectoderm, which gives rise to PGCs. Furthermore, epiblast cells isolated from e5.5 mouse embryos fail to form PGCs, unless co-cultured with either feeder cells or in the presence of the visceral

endoderm and extraembryonic ectoderm (de Sousa Lopes et al., 2004; Pesce et al., 2002; Yoshimizu et al., 2001).

1.3.1.3 Bone morphogenic proteins (BMPs)

The family of bone morphogenic proteins (BMPs) are members of the TGFβ superfamily. They function as dimers that bind and act through heterodimeric receptor complexes, and activate downstream Smads to illicit their signalling response, resulting in alterations to gene expression (Shi and Massague, 2003). The BMPs were first implicated in germ cell commitment when it was found that germ cells failed to form in *Bmp4*-/- embryos (Lawson et al., 1999), while embryos heterozygous for a mutation in *Bmp4*, display a 50% reduction in PGC number (Lawson et al., 1999). Bmp4 may act together with other family members such as Bmp8b (Ying et al., 2001), as both Bmp4 and Bmp8b have a similar expression pattern in the extraembryonic ectoderm and both *Bmp8b* and *Bmp4* mutant mice exhibit a similar PGC phenotype. In experiments where epiblast cells from e6.0 were cultured in the presence of feeder cells which expressed Bmp4, Bmp8b or a combination of both, Bmp8b rescued the *Bmp8b* deficient epiblast phenotype, while culture of *Bmp4*-/- epiblasts with Bmp4 did not rescue the phenotype, suggesting that either Bmp4 induces PGC competence within an earlier time window or that an interaction with other molecules is required (Ying et al., 2001). Bmp4 has since been shown to induce a PGC phenotype in a subset of epiblast cells when cultured at e5.5-6.0, and may induce early PGC proliferation (Pesce et al., 2002). Bmp2, derived from the visceral endoderm has also been shown to influence specification of germ cells and is additive to Bmp4 (Ying and Zhao, 2001). Bmp downstream signalling molecules, Smad1 (Tremblay et al., 2001b) and Smad 5 (Chang and Matzuk, 2001) are also important for development of PGCs. It appears that the actions of the Bmps may be mediated within the visceral endoderm. If the Bmp receptor, *Alk2* is absent in the visceral endoderm at e5.5-e6.0, PGCs do not form, while the expression of a constitutively active *Alk2* in the visceral endoderm, but not the epiblast, results in formation of PGCs in mice deficient in Bmp4 (de Sousa Lopes et al., 2004). This suggests that Bmp4 signalling within the visceral endoderm is important for the

production of an unknown factor or factors which induce PGC formation (Figure 1-8).

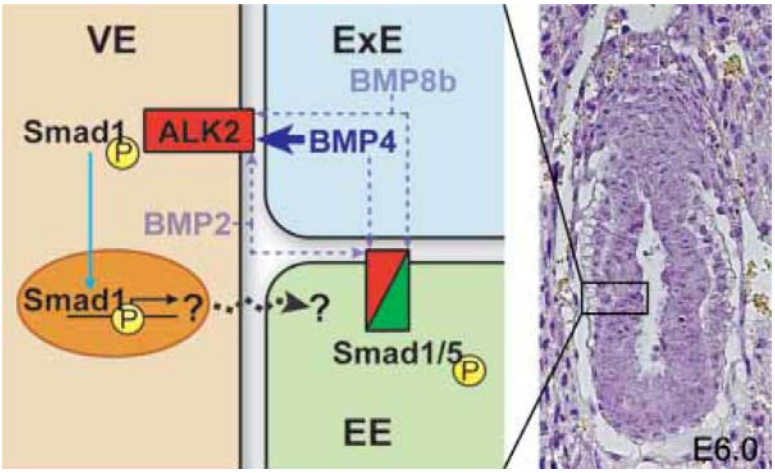

Figure 1-8. Schematic diagram showing the possible BMP4 signalling system that operates to induce PGCs in the mouse. BMP4 is produced from the extraembryonic ectoderm (ExE), and signals through the Alk2 receptor expressed on the visceral endoderm (VE). This results in phosphorylation of Smad1, which translocates to the nucleus to bring about transcriptional changes resulting in the production of an unknown paracrine molecule which acts on the epiblast (EE) to induce PGCs, possibly via Smad 1/5 signalling. From de Sousa Lopes et al., 2004

A recent study suggests that the visceral endoderm acts between e6.25-e7.25 to regulate the number of germ cells specified, either through promotion of proliferation or prevention of cell death, and thereafter is not required. The extraembryonic ectoderm appears to have an independent role in restricting the localization of PGC precursors to the posterior part of the embryo (Chuva de Sousa Lopes et al., 2007).

1.3.1.4 The PGC precursor population

In response to signals derived from the extraembryonic ectoderm, a PGC precursor population is induced to form between e6.25-e7.25. Initially within the embryonic ectoderm, there is an upregulation of *Fragilis* (Saitou et al., 2002; Tanaka and Matsui, 2002), a putative interferon-inducible gene encoding a transmembrane protein (Lange et al., 2003). *Fragilis* expression is absent from the epiblast in *Bmp4*-

/- embryos (Saitou et al., 2002). In a subset of these *Fragilis* positive cells, approximately an initial six cells gain expression of the transcriptional repressor *Blimp1* (Ohinata et al., 2005). Cells which express both *Blimp1* and *Fragilis* subsequently express *Stella* (Figure 1-9) (Hayashi et al., 2007). Despite *Stella* being one of the first genes to be expressed in PGCs, *Stella* knock-out mice display no defects in PGC induction and have normal fertility (Bortvin et al., 2004).

One gene that is essential for germ cell development is the RNA binding protein Nanos3. In Drosophila expression of *nanos* is essential for successful germ cell development (Wang and Lehmann, 1991; Forbes and Lehmann, 1998; Kobayashi et al., 1996). In the mouse there are three Nanos genes. Nanos3 is only expressed in the germ cells after e7.25 (Yabuta et al., 2006) and *Nanos3*-/- mice display a complete loss of germ cells in both males and female (Tsuda et al., 2003); the protein appears to maintain the germ cell lineage through the suppression of apoptosis (Suzuki et al., 2008).

Once the PGCs form, they migrate out of the primitive streak and are found within the extraembryonic mesoderm at the base of the allantois, where they exist as a tight cluster of cells (Ginsburg et al., 1990). In addition, cell-cell contact appears to be important for specification, as inhibiting the functions of the cell-adhesion molecule E-Cadherin, prevents PGC specification from occurring (Okamura et al., 2003). By e7.25 the cluster of around 40 PGCs found within the base of the allantois can be identified by alkaline phosphatase staining (Ginsburg et al., 1990). One intriguing question is how the six cell present at e6.25, can increase to around forty cells in just one day. The large increase in PGC number in such a short period has been attributed to either the PGC precursors altering their cell cycle, or more feasibly, through the recruitment of local cells into the germ cell lineage (McLaren and Lawson, 2005).

1.3.1.5 Expression of pluripotent genes in PGCs

At the early stages of germ cell development, a number of pluripotency-associated genes are expressed within the germ cells in mice. These include the Pou domain transcription factor, *Oct4* (*Pou5f1*) (Nichols et al., 1998), *Nanog* (Chambers et al., 2003) and high mobility group (HMG) domain containing family member, *Sox2* (Avilion et al., 2003).

In the mouse blastocyst, *Oct4* is initially expressed throughout the epiblast but gradually becomes restricted to the PGCs by e7.5 (Scholer et al., 1990; Yeom et al., 1996). The functional role of *Oct4* in developing germ cells is unknown, but without it, germ cells fail to survive (Kehler et al., 2004). *Nanog* is also expressed in mouse PGCs, where it is upregulated at e7.75 (Yamaguchi et al., 2005). Nanog is essential for germ cell development and in chimeric mice created from *Nanog* null and wild-type ES cells, although *Nanog* null cells were initially recruited to the germline, they were lost by e11.5 (Chambers et al., 2007).

In the mouse *Sox2* expression has also been shown to be restricted to the germ cell lineage where it is expressed at e6.75 (Yabuta et al., 2006; Western et al., 2005; Perrett et al., 2008). In ES cells, Sox2 and Oct4 act synergistically to drive the expression of a number of pluripotency genes (Okumura-Nakanishi et al., 2005), and Oct4/Sox2 heterodomers have been shown to bind to the *Nanog* promoter (Rodda et al., 2005). *Sox2* null epiblasts do not survive, and ES cells from *Sox2* null blastocysts cannot be obtained (Avilion et al., 2003).

In contrast to mice, SOX2 is not expressed in germ cells of the human fetal gonad (de Jong et al., 2008; Perrett et al., 2008), although it can be detected within the inner cell mass, embryonic stem cells and embryonal carcinoma cells of the human. It has been suggested that expression of SOX2 ceases either soon after or before PGC specification (Perrett et al., 2008). Another member of the SOX family, SOX17 has been shown to be present and highly expressed in germ cells within the human fetal

gonad and also germ cell tumours (de Jong et al., 2008) and this protein may therefore fulfil the role ascribed to Sox2 in mice.

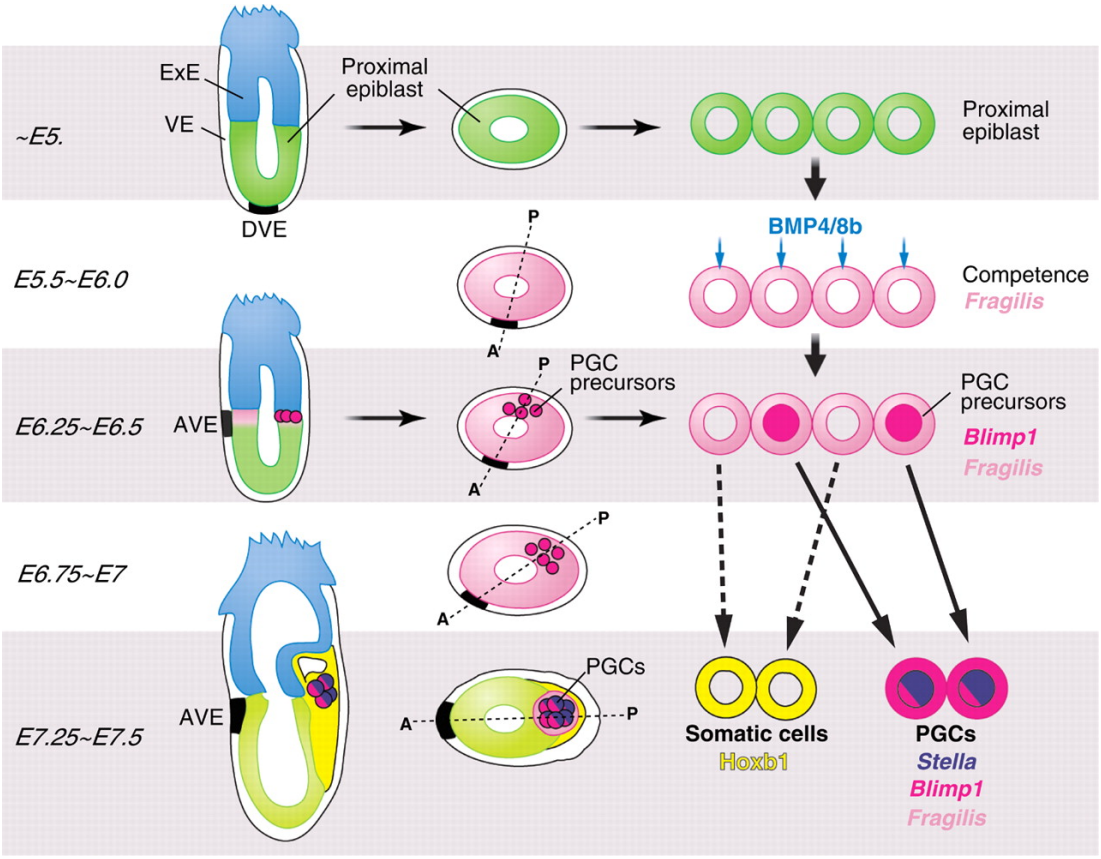

Figure 1-9 Development of germ cells in early postimplantation mouse embryos from e5.0 to e7.5. Proximal epiblast cells respond to signals from the extraembryonic tissues, by inducing the expression of Fragilis within the epiblast. A subset of these cells then expresses Blimp1. After gastrulation PGC precursors locate to the posterior proximal regions and form the founder Stella positive population. From Hayashi et al., 2007.

1.3.1.6 Blimp1 suppresses the somatic cell fate

Blimp1 is a Kruppel-like zinc-finger containing a DNA binding transcriptional repressor (Mock et al., 1996). This gene predominantly plays important roles in somatic cell specification, including the terminal differentiation of B cells into plasma cells (Shaffer et al., 2004a; Shapiro-Shelef et al., 2003; Turner et al., 1994), and the differentiation of macrophages (Chang et al., 2000). It appears to act by

repressing the transcription of genes such as *Myc* which are important for proliferation (Lin et al., 1997). Blimp1 is highly expressed in several embryonic cell types (Chang et al., 2002). Mouse embryos deficient in *Blimp1* die at e10.5, with severe placental defects, and notably these mutants lack PGCs, which appear to be lost by e7.25 (Skaznik-Wikiel et al., 2007). In the region of the proximal epiblast, cells which express *Blimp1* do not express *Hox* genes, but maintain expression of *Oct4*, *Nanog* and *Sox2* (Yabuta et al., 2006). It is thought that suppression of the somatic cell program is key to the induction of the germ cell lineage (Saitou et al., 2002), and it appears that *Blimp1* is important for this suppression. For example, at early stages of germ cell development, the *Blimp1* positive cells express *Hox* genes and mesodermal genes (Ancelin et al., 2006), the expression of these genes continues to be upregulated in the neighbouring cells, but they quickly become repressed in the *Blimp1* positive cells (Yabuta et al., 2006) (Figure 1-9). At this point there is then an upregulation of pluripotency genes such as *Sox2* (Yabuta et al., 2006) and *Nanog* (Yamaguchi et al., 2005). In *Blimp1* mutant cells, PGCs form a tight cluster and stop proliferating, they also fail to downregulate expression of *Hoxb1*, and do not show consistent upregulation of *Stella* and *Sox2* (Hayashi et al., 2007). A recent gene expression analysis of *Blimp1* deficient PGCs and their somatic cell neighbours points to Blimp1 as a critical regulator in repressing nearly all somatic genes (Kurimoto et al., 2008). The full molecular functions of Blimp1 are far from understood, but Blimp1 has been shown to interact with the protein arginine methyltransferase 5, Prmt5 (Ancelin et al., 2006) and the Prmt5 homologue, capsuleen has been shown to be required for germ cell specification in Drosophila (Anne et al., 2007), lending support to the idea that *Blimp1* is a key regulatory gene.

Interestingly, a recent report implicates another PR-domain containing transcriptional regulator, known as Prdm14, in the induction of germ cells (Yamaji et al., 2008). *Prdm14* is expressed specifically in *Blimp1* positive germ cells from e6.75. Expression of this gene is maintained in these cells until e13.5-e14.5. *Prdm14* mutants display complete germ cell loss by e12.5 and fail to show the characteristic upregulation of *Sox2* observed in normal PGCs. Mutant cells also show reduced

levels of *Stella,* although levels of *Nanog* and *Oct4* are normal. The initial activation of *Prdm14* appears to be independent of *Blimp1*, but its subsequent maintenance is *Blimp1*-dependent (Yamaji et al., 2008).

1.3.1.7 Migration of PGCs

Analysis of the migratory path taken by PGCs in the mouse embryo has been aided by the fluorescent labelling of PGCs, through the use of reporter constructs such Oct4-GFP (Anderson et al., 2000; Molyneaux et al., 2001). PGCs first migrate into the endoderm from the posterior region of the primitive streak. From between e7.5 and 8.5, PGCs move from the endoderm and become incorporated into the wall of the developing hindgut (Figure 1-10 a and b) (Anderson et al., 2000; Molyneaux et al., 2001). It is not known if colonisation of the hindgut is an active process or if it is passive, brought about by the morphogenic movements of the embryo, although at e8.5, PGCs have a non-motile morphology, a possible indication that the process is passive (Molyneaux et al., 2001). Once the PGCs enter the hindgut, it extends and closes, trapping the PGCs until e9 (Figure 1-10c). Between e9 and e9.5, PGCs begin to migrate laterally through the hindgut and dorsal mesentery (Figure 1-10d), and arrive in the gonadal ridge by e10.5 (Clark and Eddy, 1975) (Figure 1-10e). As they are migrating, they are thought to undergo 5-6 rounds of cell division, and there is an increase in the numbers of PGCs from less than 100 to approximately 4000 (Tam and Snow, 1981).

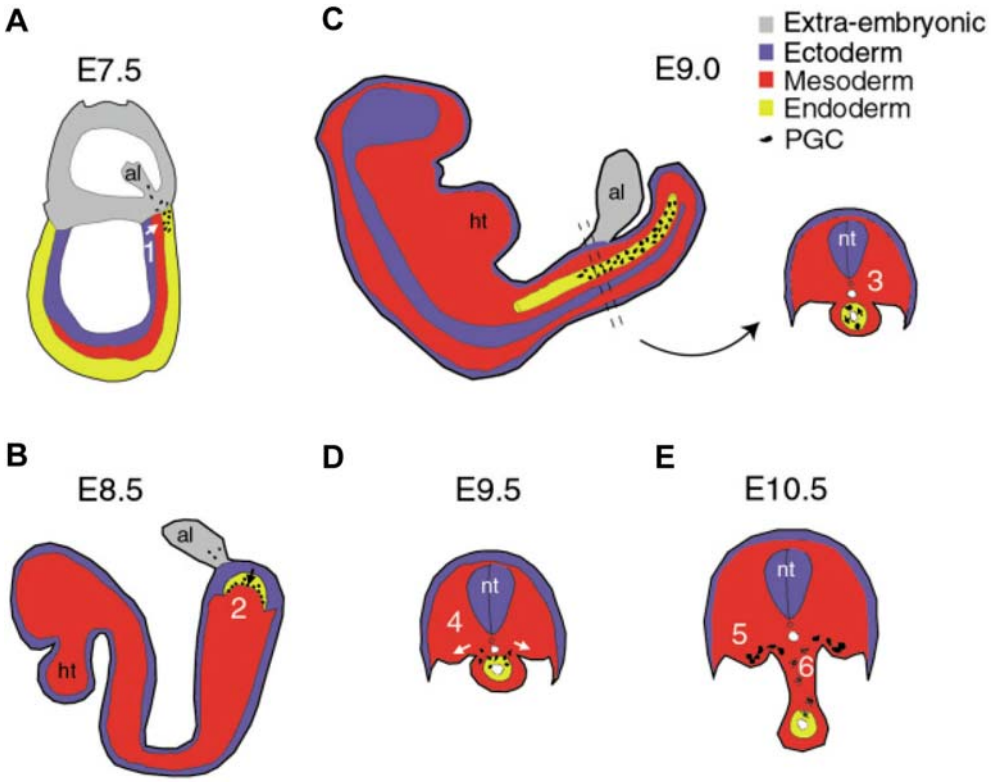

Figure 1-10 Overview of the migratory route taken by PGCs. a) PGC induction occurs within the proximal epiblast. As gastrulation occurs, PGCs move through the primitive streak and invade the definitive endoderm, parietal endoderm and the allantois b) PGCs in the definitive endoderm become incorporated into the hindgut. Some PGCs can be found remaining within the allantois c) By e9.0 the hindgut is extended and closed, PGCs are restrained within the hindgut d) Between e9.0-e9.5, PGCs emerge from the hindgut at the dorsal side and migrate to the genital ridge e) By e10.5 PGCs form a cluster of cells migrating towards the genital ridge, most of which colonize by e11.5 (Molyneaux et al., 2004)

In the human PGCs have been identified in the endoderm of the yolk sac at day 30 post-ovulation (Witschi, 1948; Freeman, 2003; Motta et al., 1997; Mc et al., 1953). During the 4th week of gestation PGCs migrate from the yolk sac along the dorsal mesentery of the hindgut (Motta et al., 1997) and enter the genital ridges by week 6 (Wartenberg, 1981).

The mechanism of PGC migration is still not fully understood and what determines the direction of travel remains unclear. Amoeboid movements are thought to be important for the active migration of PGCs, and PGCs can be identified by their possession of pseudopodia (Chiquoine, 1954). The contact which PGCs have with the surface of the cells over which they are migrating also appears to be important. It has been shown that mouse PGCs adhere to various extracellular matrix glycoproteins such as fibronectin, laminin and collagen IV and that they alter their adhesiveness to fibronectin and laminin prior to and after completion of migration (Garcia-Castro et al., 1997). Fibronectin has been shown to stimulate the migration of PGCs in explant cultures (Ffrench-Constant et al., 1991). Integrins may also be important for PGC migration, as PGCs lacking β1 integrin display poor colonization of the gonad (Anderson et al., 1999).

Studies in rodents have also shown the importance for the tyrosine kinase receptor, C-kit (Kit), in early germ cell development. The *Kit* gene has been localised to the W locus, and mice homozygous for mutations in the W locus are deficient in germ cells (Chabot et al., 1988; Nocka et al., 1989). A similar phenotype results when the steel locus is mutated (*Sl*) (Mintz and Russell, 1957). Steel encodes the ligand for Kit, Stem cell factor (*Scf*) (or Kit Ligand (*Kitl*)). PGCs express Kit from e7.5, while Kitl is expressed by the somatic cells along their migratory route (Matsui et al., 1990). Kitl is required for successful culture of PGCs (De Felici and Dolci, 1991; Dolci et al., 1993; Matsui et al., 1991). Studies suggest that Kitl acts by preventing PGC apoptosis (Pesce et al., 1993).

The behaviour of early migratory PGCs can also be influenced by diffusible factors released from cultured genital ridges. Media conditioned by e10.5 genital ridges stimulates PGC migration in culture (Godin et al., 1990), as does TGFβ (Godin and Wylie, 1991). The G-protein coupled receptor Cxcr4 and its ligand Sdf1 have also been shown to play an important role in PGC migration, and are essential for colonisation of the mouse gonad (Molyneaux et al., 2003). Migratory PGCs express Cxcr4, while Sdf1 is found along the dorsal body wall. The addition of Sdf1 to

embryo cultures has detrimental effects on PGC migration, while mutations in the *Cxcr4* gene results in a reduction in the number of germ cells that colonise the genital ridge, even though normal numbers initially reach the hindgut (Molyneaux et al., 2003). BMPs may also play a role in the migration of germ cells. For example, in an *in vitro* culture system, the BMP antagonist Noggin was found to cause migratory defects, possibly through interfering with gene expression along the migratory route (Dudley et al., 2007).

1.3.2 Germ cells within the developing gonads

As germ cells enter the gonad they are initially 'bipotential' and capable of developing down the male or female lineage, however contact with the somatic cells causes their fates to diverge.

1.3.2.1 Epigenetic alterations

Between e10.5 and e12.5, once murine germ cells have entered the genital ridge, they undergo epigenetic reprogramming, with genome-wide demethylation occurring (Hajkova et al., 2002; Maatouk et al., 2006). In females, X chromosome reactivation is completed at around the same time (Sugimoto and Abe, 2007). Reestablishment of the sex-specific methylation patterns occurs several days later in the male, but not until postnatal life in the female (Hajkova et al., 2002; Maatouk et al., 2006).

1.3.2.2 Germ cells in the developing testis

In the male, germ cells enter the gonad and become enclosed within the differentiating Sertoli cells forming the testicular cords, thereby separating the germ cells from the interstitial compartment. Germ cells at this stage of development have been extensively studied in the mouse and work performed in our lab (Gaskell et al., 2004) and by others (Honecker et al., 2004) has already demonstrated there are several key differences between the subsequent pattern of germ cell development in the mouse and human.

1.3.2.2.1 Mouse

In fetal life, gonocytes within the mouse testis, are morphologically similar with a large nucleus and small cytoplasm. The initial populations that colonise the genital ridge express both *Oct4* and *Nanog* (Rosner et al., 1990; Yamaguchi et al., 2005). Upon entry into the genital ridge, all germ cells also activate expression of the RNA helicase, *Mouse vasa homologue (Mvh)* (Toyooka et al., 2000) and the RNA-binding protein *Dazl* (Seligman and Page, 1998). All gonocytes within the mouse testis are therefore Oct4+/Nanog+/Mvh+/Dazl+, they enter mitotic arrest at e13.5 (Monk and McLaren, 1981) and just prior to birth there is a rapid downregulation in expression of Oct4 (Ferrara et al., 2006; Zayed et al., 2007). At the end of pregnancy, the gonocytes migrate from the centre of the cords to the basement membrane forming prespermatogonia. A few days after birth the prespermatogonia resume proliferation and differentiate into spermatogonial stem cells which are responsible for the production of mature spermatozoa throughout the life of the animal (de Rooij, 2001).

1.3.2.2.2 Human

Unlike the mouse, germ cells of the human fetal testis appear as a heterogenous population of cells within one single testis cord (Fukuda et al., 1975; Gaskell et al., 2004; Wartenberg, 1976) (Figure 1-11). Fukuda et al (1975) used histological analysis to identify three different subpopulations of human fetal germ cells, which they named gonocytes, intermediate cells and prespermatogonia. The gonocytes were described as cells with a high nuclear to cytoplasmic ratio and a prominent nucleolus; intermediate cells were often found to be connected to one another though intracellular bridges; while the cells with the lowest nucleo-cytoplasmic ratio were categorized as the prespermatogonia (Figure 1-11).

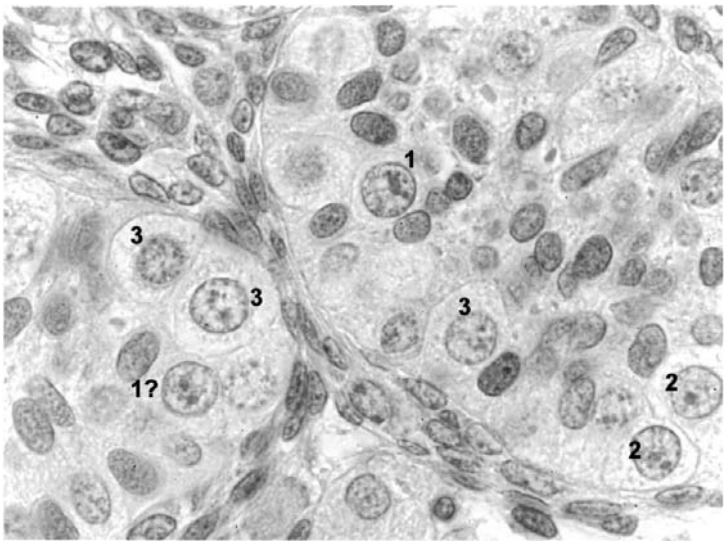

Figure 1-11 Morphological identification of germ cells in the 2nd trimester human fetal testis. Population 1: Small round cells with a high nuclear to cytoplasmic ratio. These are normally single cells in the centre of the cords. Population 2: Cells with round nuclei, often in pairs, with a large cytoplasm. Population 3: Largest of the cells, usually in groups at the periphery of the cords. From Gaskell et al., 2004

The co-existence of different subpopulations within a single testis cord has been conformed by recent studies that have found that, along with the morphological differences, human fetal germ cells also vary in their protein expression pattern. The gonocytes are immunopositive for OCT4 and KIT, and have low levels of the melanoma associated antigen, MAGE-A4. The prespermatogonia display little to no expression of OCT4 and KIT, but express high levels of MAGE-A4 protein. An intermediate population of cells with low to negative levels of OCT4 and no KIT or MAGE-A4 has also been identified (Gaskell et al., 2004). The gonocytes are the predominant cell type in the 1st trimester testis and by the 2nd trimester, gonocytes, intermediate cells and prespermatogonia are all present, with the number of prespermatogonia increasing towards the end of the 2nd trimester (Gaskell et al., 2004; Honecker et al., 2003).

1.3.2.3 Testicular germ cell tumours

Germ cells tumours are the most common solid tumour in young men between the ages of 20-40 (McKiernan et al., 1999). Testicular germ cell tumours (TGCT) can be

divided into two main histological types: the uniform seminomas and the more aggressive, nonseminomas. The vast majority of TGCTs arise from a common precursor lesion called carcinoma in situ (CIS) (Skakkebaek, 1972). CIS cells are usually located in a single row at the basement membrane of a seminiferous tubule, where they are morphologically larger and have more prominent nuclei than prespermatogonia (Holstein et al., 1987). CIS cells resemble that of human fetal germ cells (Nielsen et al., 1974) and it is widely speculated that CIS cells arise from a failure of fetal germ cells to undergo the normal maturation process, perhaps as a consequence of endocrine imbalances (Skakkebaek, 1972). Although the mechanisms are not fully understood, it has been suggested that CIS cells are fetal germ cells that have failed to differentiate normally and which therefore persist in the testis and undergo malignant transformation post-pubertally (Skakkebaek, 1972). After puberty, possibly due to hormonal changes, CIS cells begin to replicate, and as a result tubules containing only CIS cells and Sertoli cells are found in the testis (Dieckmann and Skakkebaek, 1999). The CIS cell as the origin of TGCT is strongly supported by the fact that CIS cells can be more often detected in 'at risk' populations (Giwercman et al., 1987), and that these cells are found within tissue surrounding a tumour (Jacobsen et al., 1981). Furthermore, studies have shown that over half of patients found to have CIS cells in their testes developed TGCT within 5 years (Skakkebaek, 1978).

The fetal origin of CIS is also supported by that fact that these cells express a large number of proteins in common with fetal germ cells. Proteins detectable in both fetal germ cells and CIS include: placental alkaline phosphatase (PLAP) (Giwercman et al., 1991), the most common diagnostic marker for CIS; the pluripotency markers OCT4 (Looijenga et al., 2003) and NANOG (Hart et al., 2005); and the transcription factor AP2γ (Hoei-Hansen et al., 2004; Pauls et al., 2005) all of which are found in fetal gonocytes. In addition, expression of M2A, a glycosylated monomeric sialoglycoprotein, which has an unknown function (Giwercman et al., 1988), DAZL (LitSchitz-Mercer, 2002), VASA (Honecker et al., 2004; Zeeman et al., 2002), and

the tyrosine kinase receptor KIT (Jorgensen et al., 1995) have all been reported. Table 1-1 summarizes the genes reported to be expressed in CIS.

Table 1-1 Summary of proteins expressed in embryonic stem cells (ESC), primordial germ cell (PGC), gonocytes, spermatogonial, spermatids, CIS, seminoma (SEM) and non-seminoma (N-SEM). Adapted from Rajpert-De Meyts, 2006

Protein/antigen (gene)	ESC	PGC	Gonocytes	Sp-gonia	Sp-cytes	Sp-tids	CIS	SEM	N-SEM		SpSEM
									EC	TER	
NANOG	+	+	+	–	–	–	+	+	+	–	–
OCT3/4 (*POU5F1*)	+	+	+	–	–	–	+	+	+	–	–
AP-2γ (*TFAP2C*)	+	+	+	–	–	–	+	+	+	–/+	–
TRA-1-60	+	+	+/–	–	–	–	+/–	+/–	+	–	–
PLAP *(ALPL)*	–	+	+	–	–	–	+	+	+/–	–	–
M2A *(PDPN)*	?	+	+	–	–	–	+	+	–	–	–
KIT	+	+	+/–	–/+	–	–	+	+	–	–	–
DAZL1	?	+	+	+/–	+	–	+	+/–	–	–	?
VASA	?	+/–	+/–	+	+	+	+	+/–	–	–	+
Hiwi	?	+	+	+	+	+/–	+	+/–	–	–	?
TSPY	?	?	+	+	–	–	+	+	–	–	–
Cyclin D2 (*CCND2*)	?	?	+	–	–	–	+	+/–	+/–	+/–	+?
MAGE-A4	?	–	+	+	+/–	–	+/–	+/–	–	–	+
NY-ESO-1	?	–	+	+	+	–	+/–	–	–	–	+/–

EC, embryonal carcinoma; ESCs, embryonic stem cells; N-SEM, non-seminoma; PGC, primordial germ cells; SEM, seminoma; Sp-cytes, spermatocytes; Sp-gonia, spermatogonia; SpSEM, spermatocytic seminoma; Sp-tids, spermatids; TER, teratoma.
A strong expression is marked by +, a heterogeneous expression by +/-. A minus sign means that a protein is not detectable by immunohistochemistry, but it may be present in a given cell type in extremely low quantities, and the gene may be highly expressed at the RNA level. A question mark means that there is no information concerning the protein presence. Modified and updated from Rajpert-De Meyts *et al*. (2003a).

An understanding of the mechanisms responsible for the malignant transformation of fetal germ cells has been difficult, and has been substantially hindered by the lack of an animal model. The protracted time period over which human fetal gonocytes differentiate into prespermatogonia, with the existence of mixed populations of cells, some of which express 'pluripotency' genes is thought to perhaps be partly responsible (Almstrup et al., 2006; Gaskell et al., 2004). Furthermore, although it is apparent that Sertoli cells and Leydig cells are important mediators of this maturation process, through the provision of paracrine and hormonal support, the full range of factors in the somatic- germ cell dialogue is not fully understood. Any disturbances to this support, due to genetic or environmental factors, could contribute to delayed

or arrested germ cell development and persistence of immature proliferating germ cells (Almstrup et al., 2006).

1.3.2.4 Germ cells within the developing ovary

In the female, pre-meiotic germ cells within the ovary are referred to as oogonia and nests of syncytial germ cells form surrounded by a network of stromal cells (McNatty et al., 2000). In mice, the oogonia continue to proliferate until e13.5, in a similar manner to the gonocytes (Monk and McLaren, 1981). However, oogonia enter meiosis from e13.5, and thereafter are termed oocytes (Figure 1-12) (McLaren and Southee, 1997); most oogonia have entered meiosis by e15.5, although there is evidence that a small population of germ cells may not enter meiosis until after birth (Hirshfield, 1992). Oocytes progress through leptotene, pachytene and zygotene stages of meiotic prophase and begin to enter meiotic arrest at the diplotene stage by e17.5, with the majority reaching diplotene by 5 days post-partum (Borum, 1961). As the germ cells enter meiotic arrest, they become surrounded by somatic cells and form primary follicles (Hirshfield, 1992). After puberty, meiosis is not resumed until just before ovulation. In the human, meiotic entry in the fetal ovary begins at 11 weeks gestation (9 weeks post-conception) (Bendsen et al., 2006). In the human this occurs over a longer period of time, with many oogonia continuing to proliferate, while others enter meiosis.

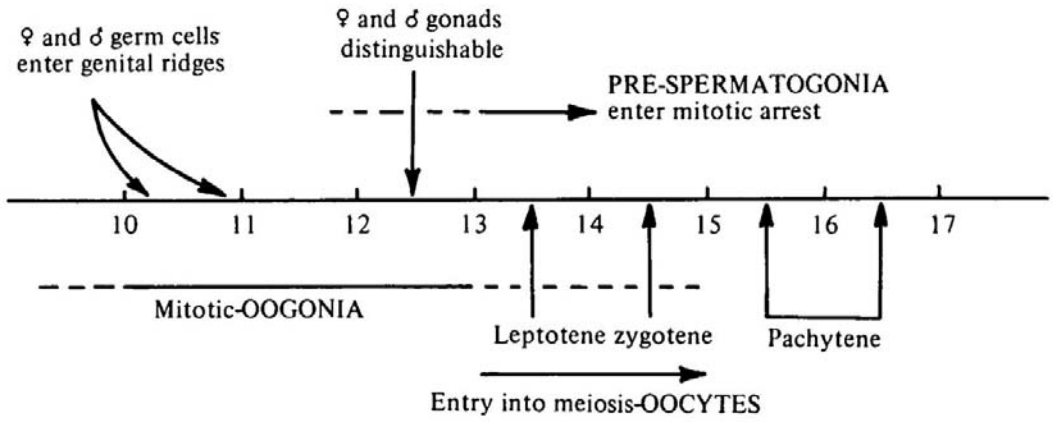

Figure 1-12 Timeline illustrating meiotic progression of female germ cells and mitotic arrest of male germ cells in the mouse. From Monk and McLaren, 1981

As germ cells enter meiosis *Oct4* expression is downregulated (Pesce et al., 1998). *Sycp3* is a meiosis specific gene, which is essential for the synapses of homologous chromosomes (Dobson et al., 1994). As *Oct4* expression is downregulated, *Sycp3* is upregulated. This occurs in a rostrocaudal manner between e13.5-e15.5 (Bullejos and Koopman, 2004). Also, the premeiotic marker stimulated by retinoic acid (*Stra8)* and the meiotic recombinase gene *Dmc1* have been shown to be upregulated along the axis of the gonad as meiosis is occurring, in a similar manner to *Sycp3* (Menke et al., 2003).

1.3.2.5 Control of meiotic entry

The decision as to whether the germ cell enters meiosis or undergoes mitotic arrest, is not an intrinsic property of the germ cells dependent upon their genetic sex, instead the process of meiotic entry is tightly regulated by the somatic cells. Experiments using XX-XY chimeric mice have shown that XX germ cells can develop as prespermatogonia and conversely, XY germ cells can enter meiosis and develop down the oocyte lineage (Palmer and Burgoyne, 1991). Similar results have been shown in experiments using XX sex-reversed male mice, in these animals Sertoli cells form in the gonad and the germ cells develop down the male lineage (McLaren, 1981). As it is the somatic cells that control the fate of the male and female germ cells, then the obvious question is whether meiotic entry in the female is initiated by the ovarian somatic cells, or whether meiotic entry is an intrinsic property of all germ cells, and that the somatic cells of the testis play an active role in the prevention of meiotic entry. The latter is favoured and the 'cell-autonomous hypothesis' suggests that germ cells have an intrinsic clock, possibly using a cell cycle counting mechanism, to prime entry into meiosis at e13.5 by default (McLaren, 1995).

The examination of germ cells that have failed to migrate to the genital ridges has also revealed that germ cells in the male embryo are capable of meiotic entry prior to birth (McLaren, 1983). Growing oocytes have also been found in the developing adrenal gland of both male and female mice after birth (Zamboni and Upadhyay, 1983). Thus if a substance is stimulating germ cells to enter meiosis then it would

have to be present in the female genital ridge, within the mesonephric region as well as in the developing adrenal gland. Furthermore, when both female and male germ cells are removed from an e11.5 gonad and grown in lung aggregates, they enter meiosis (McLaren and Southee, 1997), suggesting that if a meiosis inducing substance exists, it must also be expressed in the lung.

Experiments have also shown that there appears to be a critical window during development when germ cells are committed to the male lineage and are no longer capable of meiotic entry. For example, male germ cells enter meiosis when removed at e11.5 from the gonad and grown in reconstituted ovaries (Adams and McLaren, 2002) or *in vitro* (Nakatsuji and Chuma, 2001), but if they are recovered at e12.5 they do not enter meiosis (Adams and McLaren, 2002). This implies that at approximately e12, the male genital ridge produces a factor which programmes male germ cells, committing then to the male lineage, and inhibiting them from entering meiosis (Adams and McLaren, 2002). The commitment of female germ cells to oogenesis appears to occur later. Female germ cells at e12.5 aggregated with a male somatic environment, will develop down the male lineage, however by e13.5 they are committed to the female lineage (Adams and McLaren, 2002). This suggests that any short-range meiosis inhibiting factor must be produced by the somatic cells in the male genital ridge between e11.5-e12.5. The activity of such a meiosis initiating substance appears to be prevented if the somatic cell contacts are disrupted between e11.5-e12.5 (McLaren, 1995; McLaren and Southee, 1997).

Alternatively, support for the existence of a meiosis initiating substance is provided by the fact in co-culture experiments, ovaries containing germ cells progressing through meiosis, can induce meiosis in fetal testis (Byskov and Saxen, 1976), while conditioned media from adult ovaries or testis has been shown to induce meiosis in mouse fetal testis (Byskov et al., 1993; Byskov et al., 1998). There is therefore a need for further work to resolve apparent inconsistencies in findings.

Recently it has been reported that a conserved transmembrane protein, may also play have a role in committing germ cells to the male lineage. Sdmg1 is specifically upregulated in the Sertoli cells of embryonic testes at e12.5, where it is localised to the endosomes. Also upregulated in the developing testis are the secretary SNARES, vesicle associated membrane protein 8 (Vamp8) and syntaxin 2 (Stx2) (Best et al., 2008). *In vitro* data shows that knock-down of *Sdmg1* results in mislocalisation of Stx2 from the plasma membrane. This has led the authors to speculate that an unknown substance committing the germ cells to the spermatogenic lineage may be being secreted by the Sertoli cells at this time, aided by Sdmg1. If secretion from the Sertoli cells is inhibited at e12.5, it results in germ cells undergoing male-to-female sex reversal adding further insight into this argument (Best et al., 2008).

1.3.2.5.1 Retinoic acid (RA) signalling

Retinoic acid (RA) is the biologically active derivative of retinol (Vitamin A) (Blomhoff and Blomhoff, 2006). RA has various effects on cells and tissues during development and differentiation (Mendelsohn et al., 1992; Niederreither et al., 2002; Wendling et al., 1999). Retinol is obtained from the diet and stored mainly in the liver (Jacobsen et al., 1994), it circulates in the blood bound to retinol-binding protein 4 (RBP4). Target cells take up retinol through the RBP4 receptor, Stra6 (Kawaguchi et al., 2007) and within the cytoplasm of target cells, retinol binds to retinol binding protein 1 (RBP1). Subsequently, retinol dehydrogenase 10 (RDH10) metabolises RA into retinaldehyde (Ral) (Cammas et al., 2007). A further oxidative reaction by the retinaldehyde dehydrogenase enzymes (Aldh1a 1,2 and 3) converts Ral to RA (Maden, 2007; Reijntjes et al., 2005). The pathway leading to the production of production of RA and its subsequent receptor interaction is illustrated in Figure 1-13.

RA enters the nucleus by binding to CRABP2 (Budhu and Noy, 2002) and then is capable of binding to two families of steroid/thyroid hormone nuclear receptors, the RA receptors (RAR), of which there are 3 isoforms RARα, RARβ and RARγ. These

receptors act as heterodimers with the retinoid X receptors (RXRs), which also have 3 isoforms RXRα, RXRβ and RXRγ. Ligand-bound receptors bind as RAR/RXR heterodimers to the polymorphic cis-acting response elements of target genes or RA response elements (RARE) (Chambon, 1995; Lohnes et al., 1992) (Figure 1-13).

RA signalling can also be modulated by three RA metabolising enzymes known as Cyp26A1, Cyp26B1 and Cyp26C1 (Reijntjes et al., 2005). These enzymes metabolize RA into hydroxylated polar derivatives that do not bind the receptors (Fujii et al., 1997) (Figure 1-13).

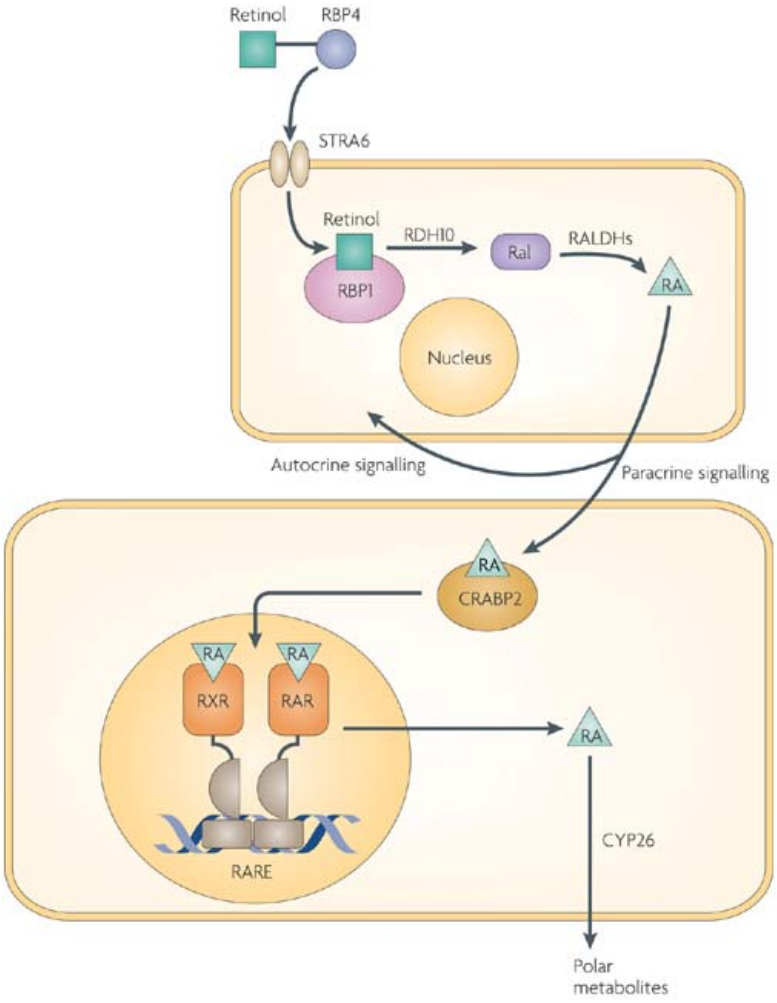

Figure 1-13 Summary of RA production, signalling and metabolism (from Maden et al, 2007)

All of the RARs and RXRs have been localised to cells within the postnatal and adult testis of the rodent (Dufour and Kim, 1999; Vernet et al., 2006). The only study documenting the cellular location of the RA receptors on the fetal testis has been performed in the rat. Rarα and Rarβ have been immunolocalised to the interstitial tissue from e14.5 and the gonocytes from e20.5. Rarγ protein was not expressed until postnatal stages. Rxrα was immunolocalised to the gonocytes from e13.5 and in the Leydig cells at e16.5. Rxrβ protein was detected in the Leydig cells throughout development and Rxrγ was expressed in a variety of cells from e20.5 (Boulogne et al., 1999).

1.3.2.5.2 The potential role of RA as a factor responsible for the initiation of germ cell meiosis

In the female mouse gonad, expression of *Stra8* is upregulated one day before meiotic entry (Menke et al., 2003). In the male, *Stra8* is absent from the embryonic testis, and is expressed in postnatal premeiotic germ cells (Menke et al., 2003). In *Stra8*-deficient ovaries, germ cells develop normally until e13.5, but they fail to initiate meiotic prophase, and deteriorate (Koubova et al., 2006). These germ cells appear to arrest before the onset of DNA replication in premeiotic S phase (Koubova et al., 2006; Baltus et al., 2006), although recent reports suggest that in the testis, *Stra8* is not required for preleptotene cells to undergo DNA replication and progress into meiotic prophase (Anderson et al, 2008. Mark et al, 2008). *Stra8* is a member of a gene family which has been shown to respond to RA. The observation that *Stra8* is required for meiotic initiation has led researchers to look at any possible effects of RA on fetal germ cell fate.

Studies by Koubova et al in 2006 found that when e11.5 ovaries were cultured in the presence of an antagonist (BMS-204493) that targets all forms of the RA receptors, for 2 days, *Stra8* expression was not upregulated, while Stra8 expression in controls was, and conversely when cultured in the presence of RA, *Stra8* was upregulated, specifically in the germ cells. In 2006, Bowles et al. reported that that the enzyme *Cyp26b1* was initially expressed in the gonads of both sexes, but became male-

specific by e12.5. In males, this enzyme expressed in the testis cords, most probably in the Sertoli cells, and was most abundant at e13.5. Using both a mouse strain where LacZ expression was under the control of a RA-response element, and a RA reporter cell line, they showed significant amounts of RA were produced by the mesonephros. In addition, they found high levels of expression of the RA producing enzyme *Aldh1a2* expressed within the mesonephroi, but not the gonads, from e10.5-e13.5. In ovarian cultures, treatment with RA resulted in a decrease in *Oct4* expression but an increase in *Stra-8*, and in contrast female gonads cultured in the presence of the RA receptor antagonist AGN193109 displayed a downregulation of meiotic genes, and a prolonged *Oct4* expression. While treatment with the cytochrome p450 inhibitor ketoconazole, increased expression of meiotic genes in the testis, and decreased expression of *Oct4*. Based on these results, Bowles et al (2006) proposed a model whereby RA, released locally from the mesonephros, diffuses into the ovary and induces germ cell meiosis through the upregulation of *Stra8*. In the male, RA cannot reach the testis because of the expression of the RA metabolising enzyme Cyp26b1 within the testis cords and therefore meiotic entry does not occur (Figure 1-14).

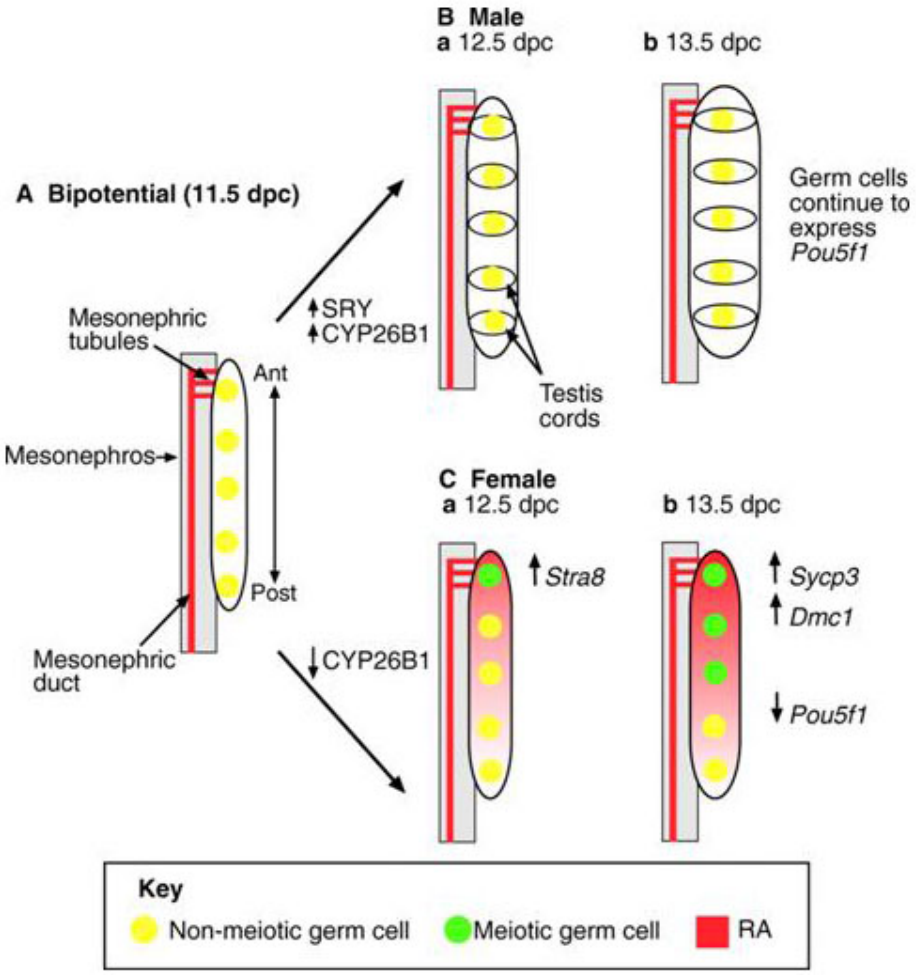

Figure 1-14 Diagram illustrating the bipotential effect of RA in the mouse testis and ovary. RA from the mesonephros enters the anterior gonad via the mesonephric tubule. In the female gonad high concentrations of RA leads to the upregulation of meiotic genes such as Stra8 and Dmc1 and downregulation of Oct4 (Pou5f1), initially at the anterior end and then throughout the whole gonad. In the male the RA metabolising enzyme, Cyp26b1 present in the male gonad prevents RA from reaching the germ cells, and Stra8 is not upregulated. From Bowles and Koopman, 2007

This model has been supported by phenotypic analysis of *Cyp26b1-/-* mice (MacLean et al., 2007). In the testes of these mice, the amount of RA was 3-fold higher in wild-type littermates. At e12.5 the testis appeared normal, but by e13.5 there were a large number of apoptotic germ cells, with some germ cells at e14.5 morphologically resembling meiotic cells. Any cells which remained at e16.5 were either apoptotic or displayed signs of meiosis.

Additional complexity in the regulation of meiotic entry is provided by a recent study that highlights the role for a member of the Nanos family of genes in the inhibition of meiotic entry in male embryos. Nanos2 is expressed specifically in germ cells of the testis (Tsuda et al., 2003; Suzuki and Saga, 2008). It appears to have an indirect role in the suppression of *Stra8* and therefore prevents male germ cells from entering meiosis after *Cyp26b1* has been downregulated (Suzuki and Saga, 2008). Ectopic expression of *Nanos2* in female germ cells also induced their differentiation down the male lineage (Suzuki and Saga, 2008) (Figure 1-15).

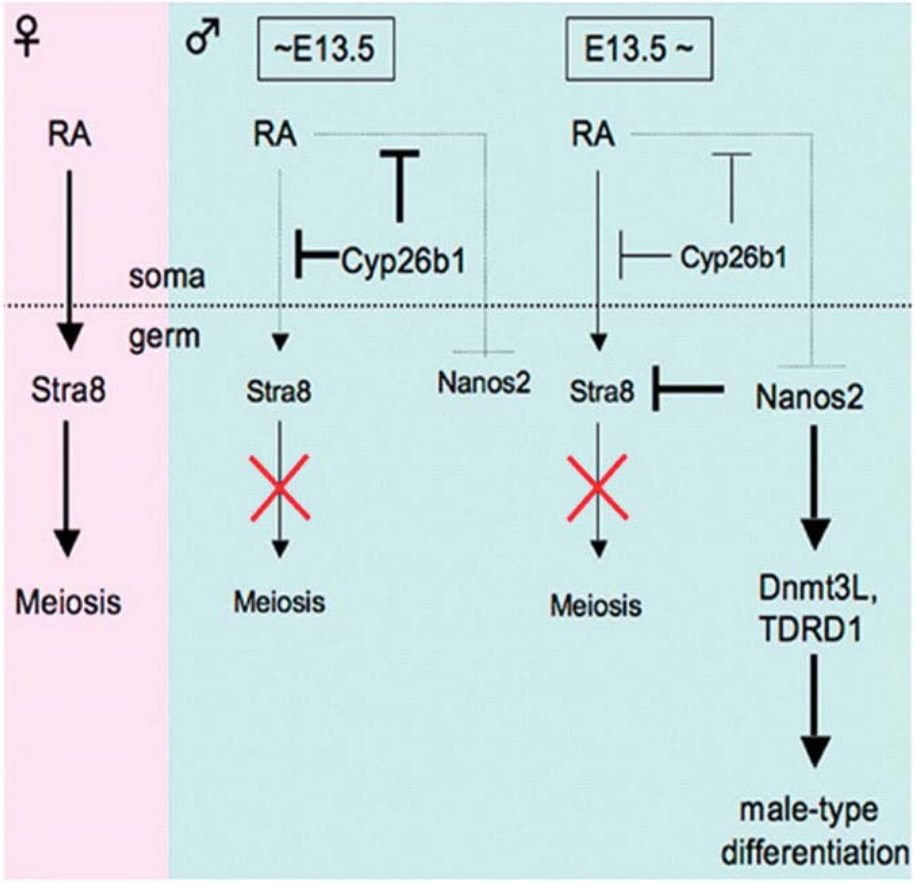

Figure 1-15 Diagram showing the potential role which Nanos2 might have in inhibiting meiosis in this male mouse embryo. At e13.5 the expression of Cyp26b1 declines in the male, but Nanos2 is upregulated and acts to inhibit Stra8, and promotes spermatogonial differentiation. From Suzuki et al., 2007

However there are some questions that remain about the role of RA in initiating meiotic entry in female. For example, experiments in the nineties studying the effects of RA on germ cells *in vitro,* never reported seeing germ cells that displayed

signs of meiotic entry. For example, Koshimizu et al in 1995 cultured both male and female mouse germ cells at e8.5, e11.5 and e13.5 in the presence of RA, and reported that RA stimulated germ cell proliferation. While in mouse fetal ovarian germ cell culture, RA was found to both stimulate germ cell division and survival, but not initiate meiosis (Morita and Tilly, 1999). The fact that RA appears to stimulate mitotic division, seems surprising when its role as a meiotic initiator would suggest that it should direct germ cells away from mitosis. Furthermore in the culture system reported by Bowles et al., 2006; Koubova et al., 2006, RA was able to initiate the expression of meiotic genes in male germ cells at e12.5, but this contradicts work by Adams and McLaren, 2002 that germ cells removed from an e12.5 gonad are already committed to spermatogenesis and are unable to initiate meiosis. In addition, all the studies to date have been exclusively conducted using mouse models and so it cannot be ruled out that alternative mechanisms operate in other species.

1.4 Paracrine signalling molecules in the testis

1.4.1 Activin

Activin is a member of the TGFβ family. The biologically active form of activin consists of two subunits covalently attached by a single disulphide bond. To date there are five isoforms of the β subunit that have been identified, but only βA and βB have been shown to dimerize, giving rise to three forms of activin: activin A, activin B and activin AB. The βA and βB subunits can also form heterodimers with an α subunit to form inhibin, which acts as an activin antagonist (Figure 1-16) (Ethier and Findlay, 2001).

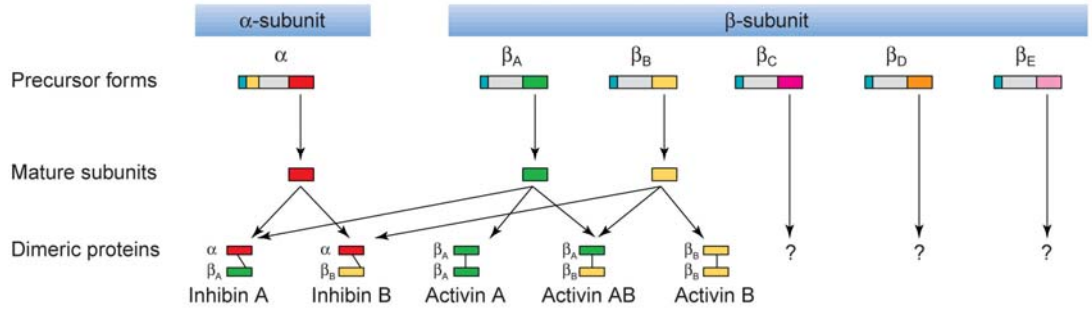

Figure 1-16 Diagram showing α and β subunit peptides. Initially the precursor forms undergo maturation to allow release of the mature C-terminal. Once mature, the β subunit can associate with an α subunit to form inhibin A or inhibin B. Alternatively the β subunits can dimerize to form activin A, B or AB. From Ethier and Findlay, 2001

Activin signals through membrane bound serine-threonine kinase receptors. Activin initially binds to a type II receptor (Figure 1-17 [1]), which recruits a type I receptor, (activin receptor IA) (Alk4) (Figure 1-17 [2]). A receptor complex forms (Figure 1-17 [3]) and the type I receptor undergoes phosphorylation (Figure 1-17 [4]), this then phosphorylates a receptor-regulated Smad, either Smad2 or Smad3 (Figure 1-17 [5]) (Massague, 1998). Once the receptor-regulated Smad is phosphorylated, it can dimerize with Smad4 (Figure 1-17 [6]), and undergo translocation to the nucleus (Figure 1-17 [7]). The Smad complex forms an association with a DNA binding partner (Figure 1-17 [8]), and binds to specific enhancers within target genes (Figure 1-17 [9]) (Massague, 1998; Massague and Chen, 2000).

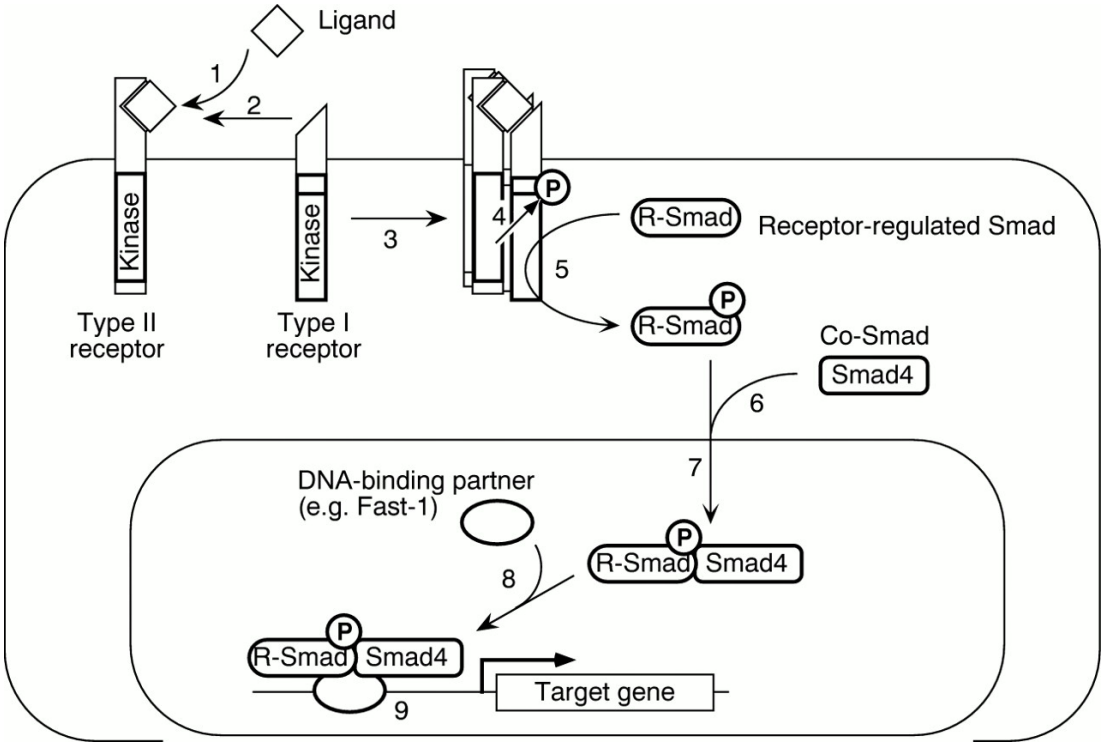

Figure 1-17 Overview of TGFβ signalling system (Massague, 1998)

In the human fetal ovary, activins may play an important role in germ cell survival (Martins da Silva et al., 2004), where it appears to act upon the somatic cell (Coutts et al., 2007a), and studies in immature mice and rats suggest that activin may play a role in Sertoli cell and germ cell survival and proliferation in the testes (Meehan et al., 2000). In a study where prepubertal rats were administered Activin A, the Sertoli cells underwent increased proliferation (Boitani et al., 1995), while *in vitro* data suggests that activin may act as a regulator of germ cell proliferation (Mather et al., 1990). Immunohistochemical staining for the activin subunits in the human fetal testis suggest that the Sertoli cells, PTM cells and Leydig all have the potential to produce activins (Majdic et al., 1997; Anderson et al., 2002). Cells in the human fetal testis also express activin receptors: ACTRIIA has been immunolocalised to the interstitium, as well as the gonocytes and some Sertoli cells; while ACTRIIB was found to be expressed in the interstitium, the gonocytes and the Sertoli cells, as well as the PTM cells (Anderson et al., 2002). This suggests that within the human fetal testis, activins have the potential to be made, and ellicit a cellular response, but as yet no specific mechanisms affected by activin have yet been identified.

1.4.2 Androgen signalling

In the developing testis, androgens are produced by the Leydig cells. These androgens are important for the masculinisation of the developing reproductive tract and external genitalia (Wilson et al., 2002). Some cells within the fetal testis express the androgen receptor (AR).

Testosterone can be converted to the more potent metabolite dihydrotestosterone (DHT) by the 5α-reductase enzyme (Russell and Wilson, 1994), of which there are two isoforms. Both testosterone and DHT exert their effects by binding the AR. The AR belongs to a nuclear receptor superfamily of ligand activated transcription factors. DHT binds AR with a higher affinity than testosterone and induces a greater response (Deslypere et al., 1992). In the unliganded state, AR primarily resides in the cytoplasm, but upon ligand binding, AR undergoes a conformational change allowing it to translocate to the nucleus (Figure 1-18). Within the nucleus the activated AR binds to androgen response elements (AREs) within the response elements of target genes to cause up or down regulation of gene expression (Shaffer et al., 2004b)

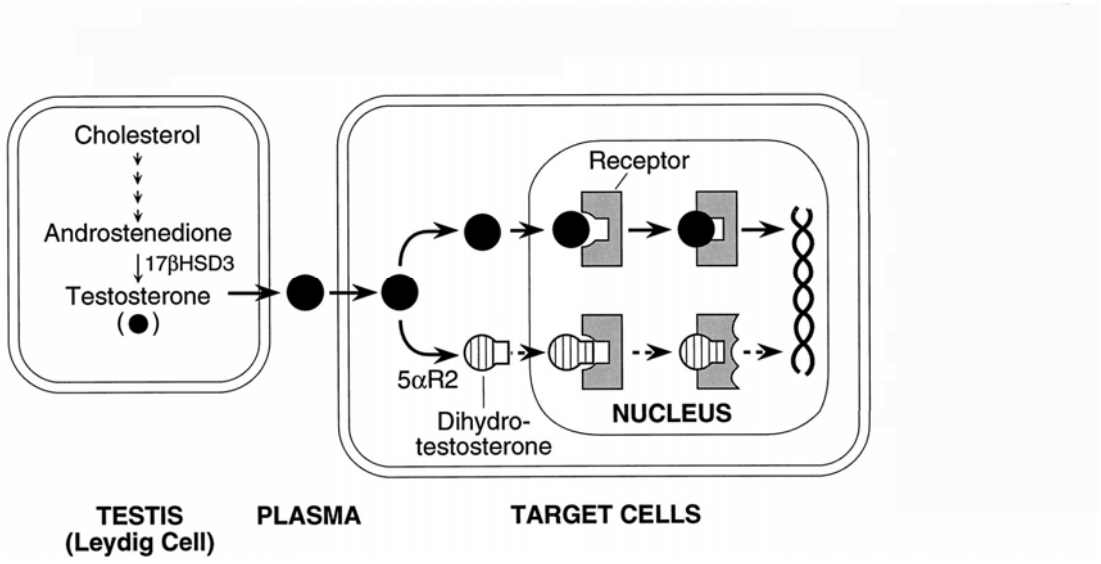

Figure 1-18 Overview of AR signalling. Modified from Wilson, 1999

In the adult testis both Sertoli cells and Leydig cells express AR, but previous work on the fetal rat (Majdic et al., 1995) and fetal human (Gaskell et al., 2004; Murray et

al., 2000) have not detected AR in either of these cell types. Instead AR is abundantly expressed in interstitial fibroblasts and PTM cells (Gaskell et al., 2004; Murray et al., 2000). Although androgen specific targets have not yet been identified in these cell types, the presence of the AR suggests that they may be important targets for the high concentrations of testosterone synthesised by the fetal Leydig cells.

1.5 Methods for studying germ cell development *in vitro*

Investigations into the molecular control of germ cell development would be enhanced if early germ cells could be obtained and reliably cultured *in vitro* in a differentiated state. Recent advances, such as the derivation of germ cells from embryonic stem (ES) cells, and the derivation of proliferative pluripotent embryonic germ cells from human fetuses are promising. Establishment of somatic cell cultures in order to study germ cell-somatic cell interaction would also be useful.

1.5.1 Embryonic stem cell derived germ cells

ES cells are pluripotent cells derived from the inner cell mass of the blastocyst (Evans and Kaufman, 1981; Thomson et al., 1998) (Figure 1-19). They undergo self-renewal *in vitro*, and when they are allowed to differentiate, they form a large variety of cell types from all 3 germ layers (ectoderm, endoderm and mesoderm).

Several groups have now reported the derivation of germ cells from ES cells for both the mouse and human. Interestingly, a variety of methods have now been used for obtaining these cells, and each method appears to present a different result. A number of laboratories have employed the use of genetically modified reporter cell lines to enhance the study of germ cell-derived ES cells.

Hubner et al (2003) were the first to report the *in vitro* differentiation of germ cells from ES cells. They used a mouse ES cell line where expression of GFP was driven by the *Oct4* promoter. These ES cells were allowed to differentiate as a monolayer.

After 8 days of differentiation, around 40% of the cells were found to be *Oct4* positive, and also expressed both *Kit* and *Mvh* and were therefore classified as germ cells. After a few weeks of culture, aggregates were found to break away from the colonies, and these aggregates were maintained in suspension for another few weeks. Within the cultures, follicle-like structures which contained cells thought to be 'oocytes' were found. These cells were surrounded by a zona pellucida (Hubner et al., 2003). Although, in a more recent analysis of a number of meiosis specific markers in germ cell-like ES cells, found that although Sycp3 was expressed, the nuclear distribution was highly abnormal, while other meiotic markers such as SYCP1 and REC8 were absent (Novak et al, 2006).

In the same year differentiation of ES cells down the male germ cell lineage was also described. Toyooka et al in 2003 used mouse ES cell lines where GFP expression was driven by the *Mvh* promoter. In their culture system ES cells were allowed to form embryoid bodies. Initially the cultures were negative for *Mvh*, but after several days, a number of GFP positive cells could be detected. Additionally, the number of GFP positive colonies was found to be enhanced by supplementing the cultures with BMP4. These cells were then aggregated with embryonic gonadal tissue and transplanted into a host, resulting in the production of haploid spermatids that were positive for the *Mvh*-GFP transgene.

Another group has shown that the formation of ES cell-derived germ cells can be enhanced by supplementing the media with RA. Geijsen et al (2004) allowed embryoid bodies to form *in vitro* for 5 days, and were then able to isolate SSEA1 positive cells; SSEA1 is expressed by undifferentiated ES cells and early germ cells. These putative germ cells were then cultured in the presence of RA, resulting in the *in vitro* expansion and they were subsequently characterised as alkaline phosphatase positive with motile characteristics, suggestive of migratory PGCs and displayed signs of imprinting erasure, similar to PGCs. These cultures were found to be capable of supporting the maturation of PGCs into haploid male gametes, which could form blastocyts when injected into oocytes. Within the cultures no oocytes-

specific genes could be detected, suggesting that this culture system could not support the generation of female germ cells.

One group has reported the production of live mice from ES cell-derived germ cells using mouse ES cell transfected with *Stra8* fused to a GFP reporter (Nayernia et al., 2006). GFP positive cells were isolated by flow cytometry and then cultured in the presence of RA. The cells were then transfected with a second fusion reporter gene, Protamine 1-ds red, in order to identify post-meiotic cells. These red cells were then isolated from the cultures and injected intracyoplasmically into mouse oocytes resulting in the production of some live offspring which died prematurely probably as a result of imprinting defects. As yet these experiments have not been repeated by any other groups.

As germ cell-derived ES cells develop, a niche may be important for their survival and differentiation. Studies have shown that the formation of male germ cells requires embryoid body differentiation and the use of a 2D culture system only results in the formation of female germ cells (Hubner et al., 2003; Kerkis et al., 2007). It has been reported that within the differentiating embryoid body, male germ cells tend to form on the surface, while female germ cells form inside the embryoid body (Kerkis et al., 2007). Directed differentiation of male germ cells has also been reported. Successful derivation of developing male germ cell occurs when ES cells are allowed to differentiate in the presence of both RA and testosterone (Silva et al., 2008) or from testicular cell conditioned media containing cytokines such as BMP4 and GDF-9 (Lacham-Kaplan et al., 2006).

Only one group thus far has reported the differentiation of human ES cells into germ cells (Clark et al., 2004). When human ES cells are induced to form embryoid bodies, the expression of OCT4 and other pluripotency genes declines, while mature germ cell markers such as *VASA* and *PUMILIO2* increase.

1.5.2 Embryonic germ cells

In addition to ES cells, another *in vitro* pluripotent cell line derived from germ cells has also been described. When PGCs are cultured under very tightly regulated culture conditions, they will form embryonic germ (EG) cells (Figure 1-19). These cells, like ES cells, are highly proliferative cells which express alkaline phosphatase, and other pluripotency markers such as *Oct4* and *Nanog*. They can also give rise to all three germ layers in a similar manner to ES cells. The derivation and long-term culture of mouse EG cells has been relatively unproblematic (Durcova-Hills et al., 2006; Matsui et al., 1992; Resnick et al., 1992; Rich, 1995; Rohwedel et al., 1996; Shim et al., 2008 Durcova-Hills et al., 2001). This is in comparison to the derivation and maintenance of human EG cell lines, where since the initial cultures were reported by Shamblott et al., in 1998, only a handful of other groups have reported their derivation and successful culture (He et al., 2007; Liu et al., 2004; Park et al., 2004; Turnpenny et al., 2003).

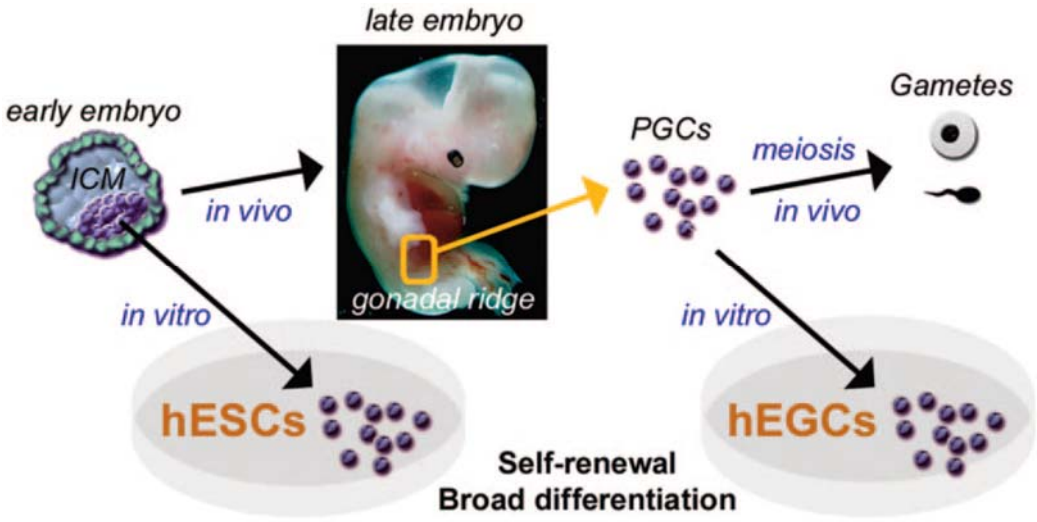

Figure 1-19 Cartoon depicting the derivation of human embryonic stem cells from the inner cell mass of a blastocyst and the derivation of human embryonic germ cells from PGCs. From Turnpenny et al., 2006

The derivation of human EG (hEG) cells involves the enzymatic and mechanical dispersion of embryonic gonads at 5-9 weeks post-ovulation, followed by their

culture in the presence of feeder layers, such as STO fibroblasts or mouse embryonic fibroblasts (MEFs). The hEG cells have been cultured in the presence of the anti-apoptotic stem cell factor (SCF), Leukaemia Inhibitory Factor (LIF), fibroblast growth factor 2 (FGF2) and forskolin. A recent study has describes the successful derivation of hEG cells on fibroblast-like cells derived from the gonadal ridges and dorsal mesenteries of 1st trimester fetuses, which endogenously expressing FGF2 and LIF (He et al., 2007). EG cells have been shown to be capable of forming embryoid bodies (He et al., 2007; Shamblott et al., 1998) and their therapeutic potential was demonstrated when Kerr et al. (2003) reported the introduction of differentiated hEGs into the cerebral spinal fluid of paralysed mice, resulting in their distribution throughout the spinal cord where they produced factors that aided the recovery of motor neurons.

EG cells have been shown to express the characteristic pluripotent cell surface markers, stage specific embryonic antigens SSEA-1 and SSEA4, and most groups report the expression of the tumour rejection antigens, TRA1-60 and TRA1-81 (Turnpenny et al., 2006). The EG cells have been shown to be capable of maintaining a normal karyotype for around 10 passages (reviewed in Turnpenny et al., 2006).

Park et al in 2004 reported the culture of hEG cells for up to a year. However subsequently, others have reported difficulty in maintaining the pluripotency of the hEG cells long-term. It has been reported that levels of OCT4 decrease with long-term passage, and with repeated freeze-thawing (Liu et al., 2004; Turnpenny et al., 2003). Although the expression of OCT4 and NANOG has been demonstrated, other genes, normally expressed at high levels in human ES cells, such as SOX2, FGF4 and FRAGILIS, have never been detected (Turnpenny et al., 2006). As SOX2 does not appear to be expressed in human PGCs (Perrett et al., 2008), and given that expression of OCT4, NANOG and SOX2 are all essential for the establishment of ES cell lines (Avilion et al., 2003), questions have been asked as to whether the absence of SOX2 hinders the long-term maintenance of hEG cells (Perrett et al., 2008). A

recent report also shows that there is a correlation between the number of OCT4 positive cells and the number of EG colonies, suggesting that the age of the fetus from which the cells are derived is critical (Kerr et al., 2007).

1.5.3 Models for studying somatic cells/germ cell interactions in the developing testis

Primary cultures of Sertoli cells, PTM cells and gonocytes have been established from a variety of species (Fritz et al., 1975; Majumdar et al., 1998; Tung and Fritz, 1980; Van Dissel-Emiliani et al., 1996). However these cultures are only maintained short-term and normal cellular interactions between differing cell types such as Sertoli cell and PTM cell cannot be achieved (Tung and Fritz, 1980). Cellular architecture and function is better maintained when testicular tissue is cultured on a reconstituted basement membrane (Hadley et al., 1985; Noguchi et al., 2006; Gassei et al., 2006). A number of laboratories have already used organ culture as a technique for studying human fetal testis and the effects of various molecules such as RA (Lambrot et al., 2006a) and environmental pollutants (Lambrot et al., 2006b Hallmark et al., 2007). But these cultures can only be maintained for a few days, before cell death occurs and the tissue architecture becomes compromised. A more promising model system for studying cellular interactions within the developing testis is xenografts. For example, when neonatal and prepubertal testicular tissue is transplanted subcutaneously into an immunodeficient host tissue function is maintained and can result in the successful completion of spermatogenesis. This technique has now successfully been performed in a variety of species, such as the mouse, rat, hamster and monkey (Gassei et al., 2006; Honaramooz et al., 2002; Schlatt et al., 2003; Schlatt et al., 2002;. This system could be a valuable model for studying germ cell-somatic cell interaction.

1.6 General aims

The overall aim of this thesis was to study germ cell development and the influence of the somatic cells. For this study, human fetal gonads were obtained during the 1st

and 2nd trimester, and additionally mouse embryonic stem cell lines were used as an *in vitro* model for the study of germ cell gene expression.

The general aims of this thesis are:

- To further characterize germ cells in the human fetal testis, by studying their pattern of protein expression during the 1st and 2nd trimester.
- To determine whether a similar model leading to the meiotic induction of germ cells in the developing ovary and inhibition of meiosis in the fetal testis operates in the human fetal gonads as has been proposed in the mouse.
- To establish cultures of human fetal testicular somatic cells that maintain a differentiated phenotype, in order to provide a model system to further understand gene expression in the 2nd trimester human fetal testis.
- Culture and differentiate mouse ES cells in order to understand further germ cell gene expression and Kit signalling in both wild-type ES cells, as well as cell lines mutant for the *Kit* receptor.

2 General materials and methods

2.1 Recovery of human fetal gonads

Human fetal testes and ovaries were obtained following elective termination of pregnancy during the first (50-65 days gestation) and second (13-20 weeks gestation) trimesters, as dated from last reported menstrual period. Women gave informed consent in accordance with national guidelines (Polkinghorne, 1989.). Ethical approval was obtained from Lothian Pediatrics/Reproductive Medicine Research Ethics Committee. No terminations were for reasons of fetal abnormality, with all fetuses used appearing morphologically normal. Termination of pregnancy was performed by medical and nursing staff at the Brunstfield Suite within the Simpson's Centre for Reproductive Health, Edinburgh Royal Infirmary. Terminations were induced with mifepristone (200mg, orally) followed by misoprostol (Pharmacia, Surrey UK; 200mg every 3 hours per vaginam). Gestational age was determined by ultrasound and for second trimester samples this was confirmed by direct measurement of foot length. The sex of first trimester fetal gonads was determined by PCR for the *SRY* gene. Testes and ovaries were carefully dissected free from the abdominal cavity and either snap frozen and stored at -80^{o}C prior to extraction of total protein or RNA, fixed for immunohistochemistry or dissociated for cell culture.

2.2 Tissue fixation and processing

Testes and ovaries were divided into two parts, and fixed in Bouins solution (Triangle Biomedical Sciences, Lancashire, UK) for 2-3 hours, transferred to 70% ethanol (Fisher Scientific) and embedded in paraffin wax by the histology support service. An automated Leica TP1050 processor (Leica Microsystems, Milton Keynes, UK) was used for processing the tissue and dehydrating through a series of graded alcohols on a 17.5 hour cycle. Processed tissue was saturated and embedded by hand in liquid paraffin wax and cooled.

Sections (5μm) were cut using a hand operated microtome (Leica RM2135), placed in a 42^{o}C water bath (Lamb RA, model E/65), and were mounted onto positively

charged microscope slides (BDH, Lutterworth, UK). They were dried overnight in a 50^{o}C oven (Lamb, RA, model E28.5), before being dewaxed in xylene (Triangle Biomedical Sciences), and rehydrated through a series of different concentrations of ethanol (95%-70%).

2.3 Tissue staining and Immunohistochemistry

2.3.1 Haematoxylin and eosin staining

Following dewaxing and rehydration, sections were then immersed in Harris's haematoxylin (Triangle Biomedical Sciences) for 4-5 minutes, which stains the nuclei blue. Sections were washed in tap water, immersed in 1% acid alcohol to remove nonspecific cytoplasmic staining and then rinsed again in tap water. Sections were then immersed in Scott's tap water for 30 seconds to allow the blue colour to develop. Sections were then immersed in eosin Y (1% aqueous solution mixed with 1% alcohol solution at a ratio of 3:1 - Triangle Biomedical Sciences) for 30 seconds to stain the cytoplasm pink, before being rinsed in tap water. Sections were then dehydrated in a series of alcohols. Slides were cleaned with xylene (2x5 minutes) and mounted with coverslips (VWR, Poole, UK.) using Pertex (Cell Path, Hemel Hempstead, UK).

2.3.2 Immunohistochemistry

Immunohistochemistry is the process whereby a specific antigen within tissues or cells is recognised by an antibody raised to an epitope within the protein of interest. The protein of interest is detected by binding of a specific primary antibody, which is then bound by a secondary antibody conjugated with a biotin molecule. Streptavidin horseradish peroxidise (HRP) binds the biotin and the complex is visualised by 3,3-diaminobenzidine tetrahydrochlorine (DAB) that turns brown in the presence of the peroxidise (Figure 2-1).

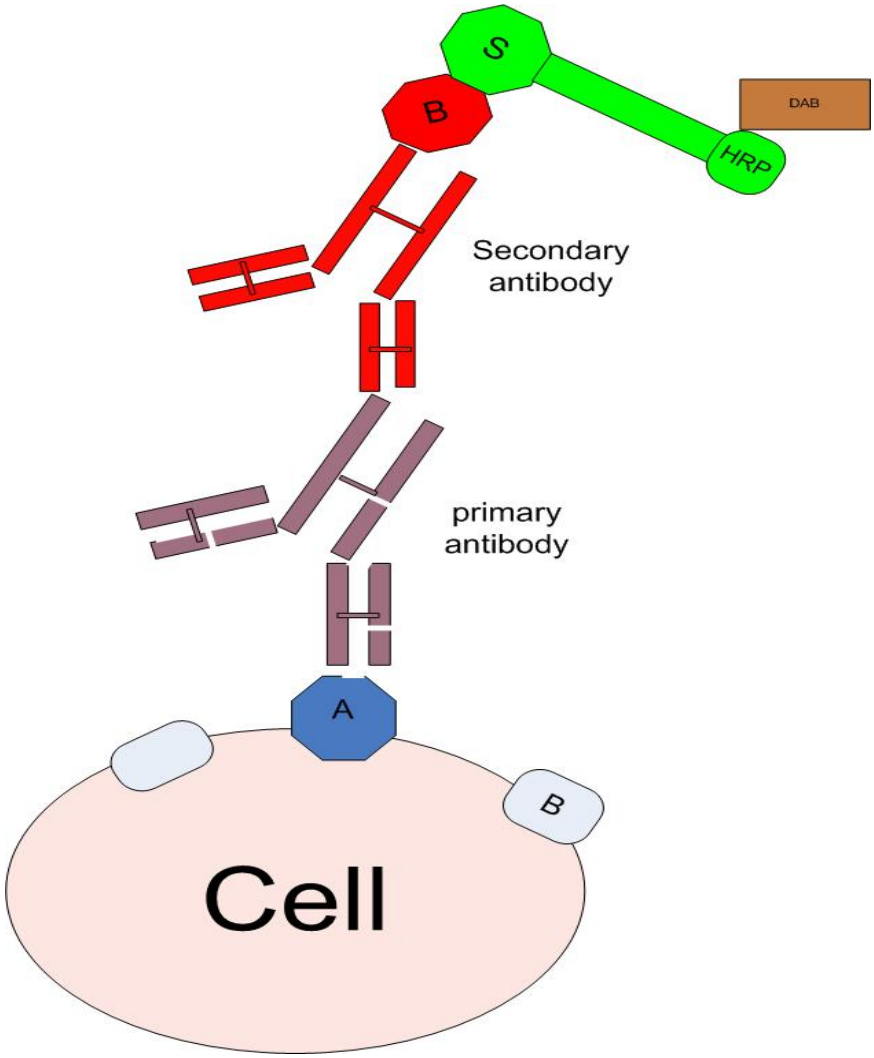

Figure 2-1 Summary of immunohistochemical detection method used

2.3.3 Antigen Retrieval

The fixation process can mask a number of antigens, due to the cross linking of a number of amino acids, and this can reduce binding by the primary antibody. By exposing the tissue to high levels of heat and altering the pH, epitopes can be exposed, allowing the antibody to bind the antigen (Shi et al., 1997). Heat-induced

antigen retrieval was performed by placing slides in a pressure cooker in 0.01M citrate pH6 for 5 minutes on full power, with 20 minutes standing time prior to cooling. First trimester tissue was pressure cooked for 3 minutes with 10 minutes standing time.

2.3.4 Methanol peroxide block

In order to block endogenous peroxidase activity, which would otherwise interfere with horseradish peroxidise amplification, tissues were incubated in 3% H_2O_2 (BDH laboratory supplies) in methanol for 30 minutes. After the 30 minutes, slides were washed by being placed in Tris-buffered saline (TBS) solution (section 2.7) for 5 minutes.

2.3.5 Serum block

Non-specific binding of secondary antibodies was prevented by incubating with the appropriate non-immune block. Serum was used from the species in which the secondary antibody was raised. For example, if the secondary antibody was raised in rabbit, tissue was incubated in TBS containing 20% normal serum (e.g rabbit) (Diagnostics Scotland, Carluke, UK) and 5% BSA (Sigma, Poole, Dorset, UK) for 30 minutes.

2.3.6 Avidin-biotin block

Some tissues have high levels of endogenous biotin, making them prone to binding high levels of the streptavidin-HRP without the addition of the biotinylated secondary antibody. An avidin-biotin blocking step was used to block endogenous biotin, which can cause non-specific binding. Immediately after the serum blocking step, tissues were incubated with avidin-D (Vector Laboratories, Peterborough, UK, 0.01M) for 15 minutes. After 2x5 minute washes in TBS, slides were incubated with biotin for 15 minutes (Vector Laboratories, Peterborough, UK).

2.3.7 Primary antibodies

Details of primary antibodies used are listed in Tables 3-2, 4-1 and 5-1. All primary antibodies were diluted in the appropriate normal serum, and incubated overnight at

4°C. Negative controls were included in each run where the primary antibody was replaced with a non-immune block. After the overnight incubation, the slides were then washed in TBS (2x5 minutes).

2.3.8 Secondary antibodies

Biotinylated secondary antibodies were diluted in their appropriate normal serum and incubated on tissue sections for 30 minutes at room temperature. Secondary antibodies used are listed in Table 2-1.

2.3.9 Developing, counterstaining and mounting

Streptavidin-HRP (DAKO, Cambridge, UK) was used, diluted 1:1000 and incubated with slides for 30 minutes at room temperature. Slides were washed for 2x5 minutes in TBS, then incubated with DAB substrate (DAKO), for 1-5 minutes, until immunopositive staining (brown) was observed. DAB produces an insoluble stable dark brown substance at the sites of antibody localisation. DAB is an electron donor that oxidises in the presence of peroxidise, resulting in a colour change. Slides were then immersed in water, counterstained with haematoxylin for 2-5 minutes, rehydrated and dewaxed, before being mounted in pertex.

2.3.10 Fluorescent immunohistochemistry

Double label fluorescent immunohistochemistry is frequently used for the cellular and subcellular colocalisation of independent antigens. Primary antibodies for double labelling should be derived from independent species. In order to use two antibodies from the same species, monovalent secondary antibodies with FAB fragments were used (Lewis Carl et al., 1993).

Fluorescent immunohistochemistry was performed in the same way as the DAB immunohistochemistry up until the secondary antibody stage, with the exception of the washes being performed in PBS 0.5% Tween (Sigma) followed by PBS for 5 minutes. Biotinylated, peroxidase or direct fluorescent conjugated secondary antibodies were used. Peroxidase and biotinylated labelled antibodies were diluted in normal serum, and direct conjugate antibodies were diluted in PBS. Peroxidase

labelled and direct fluorescent conjugates were incubated for 1 hour at room temperature. The biotinylated antibodies were incubated on the sections for 30 minutes, followed by either streptavidin 488 or 546 diluted 1:200 in PBS for 1 hour. The peroxidase antibodies were incubated for 1 hour followed by tyramide-Cy 3 or tyramide-enhanced fluorescein (PerkinElmer, Massachusetts, USA) diluted in tyramide buffer (1:5 dilution) for 10 minutes. Information regarding secondary antibodies used is listed in Table 2-1. Nuclear counterstaining was performed using DAPI (Sigma) diluted 1:1000. Slides were mounted using permaflour (Immunotech, Marseille, France).

Table 2-1 Summary of secondary antibodies used for immunohistochemsitry

Host	**Target**	**Conjugate**	**Source**	**Catalogue number**	**Dilutions**
Goat	anti-mouse	biotinylated	Sigma	B6649	1:500
Chicken	anti-rabbit	biotinylated	SCruz[1]	Sc-2986	1:500
Goat	anti mouse	peroxidase	Dako	PO447	1:200
Goat	anti-rabbit	(Fab)-biotinylated	AbCam[2]	Ab7055	1:600
Rabbit	anti-mouse	biotinylated	Dako	E0464	1:500
Goat	anti rabbit	biotinylated	Dako	E0432	1:500
Chicken	anti-mouse	biotinylated	SCruz[1]	Sc-2985	1:500
Chicken	anti-goat	peroxidase	SCruz[1]	Sc-2953	1:200
Rabbit	anti-goat	peroxidase	Sigma	A5420	1:200
Goat	anti-rabbit	alexa 488	Molecular probes[3]	A11034	1:200
Goat	anti-rabbit	alexa 546	Molecular Probes[3]	A11035	1:200

[1]Santa Cruz Biotechnology, CA, USA

[2]R+D Systems, MN, USA

[3]Molecular Probes, Leiden, The Netherlands

2.3.11 Imaging

Non-fluorescent images were photographed using a PROVIS microscope (Olympus Optical, London, UK) and a Canon DS126131 camera. Images were obtained using Canon EOS image capture software (Canon, Woodhatch, Surrey, UK).

Fluorescent images were visualized using a LSM 510 Meta-Confocal (Carl Zeiss, Hertfordshire, UK). Photographs were compiled using Adobe Photoshop 7.0 (Adobe Systems Inc, CA, USA.).

2.4 RNA analysis

2.4.1 RNA extraction from tissues and cells

RNA extraction was performed using reagents supplied in the RNA mini extraction kit (Qiagen, Crowley, UK). For RNA extraction from tissues, tissue was lysed by either grinding in liquid nitrogen or using a Qiagen Tissue Lyser. Lysates were spun through a Qiagen shredder spin column placed in a 2ml collection tube. RNA extraction was performed using a mini column, following manufacturer's instructions. On-column DNase treatment, to prevent DNA contamination, was performed (Qiagen). RNA extraction was completed by eluting RNA in a volume of RNAse-free H_20 (30µl-60µl).

2.4.2 RNA quantification

RNA concentration was determined using a Nanodrop-1000 (Labtech International, East Sussex, UK). This is a spectrophotometer which allows accurate and reproducible quantification of RNA. The concentration of RNA was adjusted to 100ng/µl in RNAse free H_20.

2.5 TaqMan® reaction

2.5.1 Principles of reaction

Quantitative PCR is a modified form of PCR, which allows quantification of the amount of RNA present in a sample. PCR should amplify the DNA exponentially, doubling the number of molecules present within each amplification cycle. Therefore, the amount of a specific cDNA present in the sample can be calculated. Real-time PCR allows a measure of the amount of DNA after each cycle of PCR, by the use of fluorescent markers.

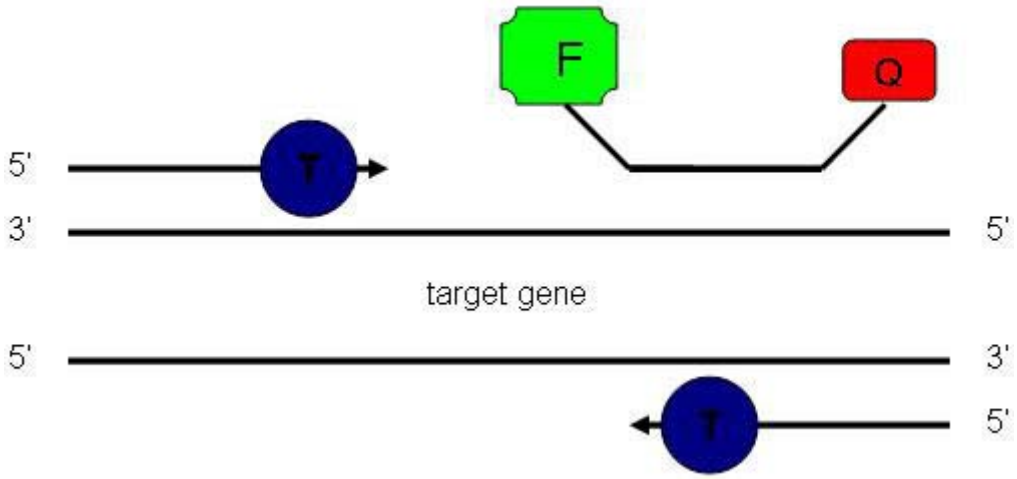

Figure 2-2 Principle of TaqMan® reaction with probe intact

In the TaqMan® reaction fluorescent probes are used, and upon melting of the DNA, the probe will bind to the complementary sequence in the region of interest in the template DNA, along with the primers. Once taq polymerase becomes activated, the polymerase starts writing the complementary strand to the primed single strand DNA (Figure 2-2). The polymerase has 5'-3' exonuclease activity, as it reaches the probe the reporter and quencher separate, resulting in an increase in fluorescence (Figure 2-3). As the reaction progresses, more and more fluorescent reporter is liberated, allowing an accurate quantification of the final, and thus the initial, quantity of DNA, through measurement of the amount of fluorescence.

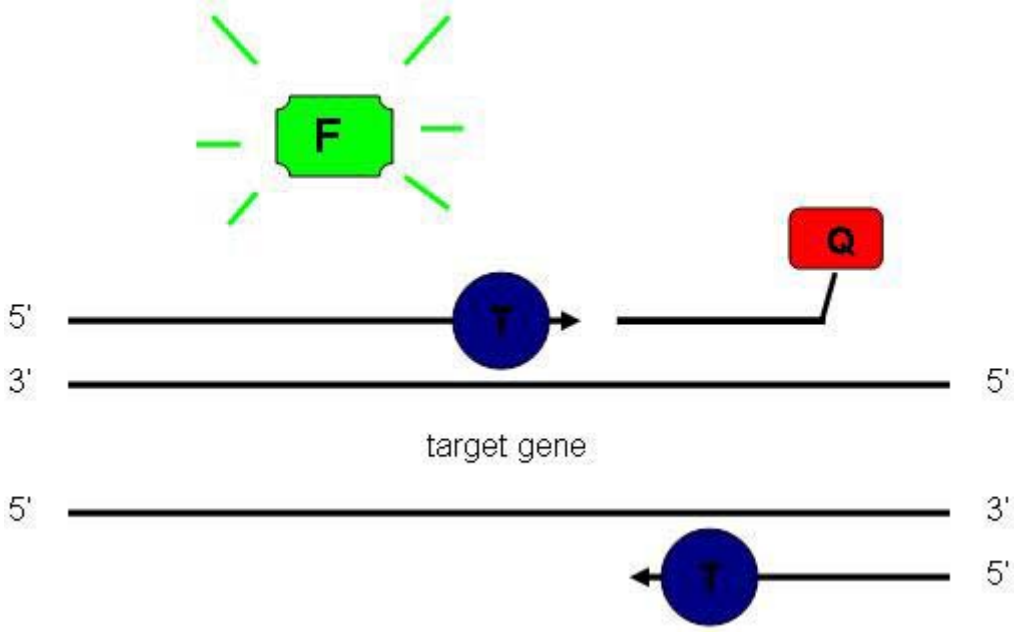

Figure 2-3 TaqMan® reaction following release of fluorescence

2.5.2 Universal probe library

The Roche Universal Probe Library™ (Roche, West Sussex, UK) consists of 165 short hydrolysis probes. The probes have the duplex stabilising DNA analogue locked nucleic acid (LNA), which allow the probe size to maintain short, while maintaining specificity and a melting temperature permitting hybridization of the probes. Probes are labelled 5' terminal with fluorescien (FAM) and the 3'-proximal terminal is labelled with a dark quencher dye. As they only bind to 8-9 nucleotides, each probe can recognize target sequences which occur frequently in the transcriptomes.

The design software at Roche Applied Science Assay Design centre (**https://www.roche-appliedscience.com/sis/rtpcr/upl/**) for each assay suggests an optimal set of PCR primers and one probe. Primers and probes used are listed in tables 4-2, 5-2 and 6-1.

2.5.2.1 Preparation of cDNA using random hexamers.

A cDNA mastermix was prepared, and each reaction was set up at 20µl. Reagents per reaction (all Applied Biosystems, California, USA) were used at the following concentrations: PCR buffer I (1x), $MgCl_2$ (5mM), dNTPs (1mM), RNase inhibitor (1µg/µl), Multiscribe reverse transcriptase (2.5µg/µl), random hexamers (2.5µM), 5µl of H_2O and 400ng of RNA. In every run, a positive, negative and no RT control was included. The reaction was run on a Biometra PCR machine set at 25°C for 10 minutes, 42°C for 1 hour, 99°C for 5 minutes and 5°C for 5 minutes.

2.5.2.2 TaqMan reaction

Each TaqMan® reaction was performed in triplicate and on a Microamp Fast Optical 96 well reaction plate (Applied Biosystems). During the course of the studies 2 sets of reagents were used. Each well was set to contain either of the following reagents:

- Universal mastermix (Applied Biosystems) (2x) 7.5µl
- 18S primer (0.02µM) primer/probe mix (0.08µM) (Applied Biosystems) 0.225µl
- Forward Primer (200nM) 0.15µl
- Reverse Primer (200nM) 0.15µl
- Target probe (100nM) 0.15µl
- H20 5.325µl
- cDNA 2µl

OR:

- 2x faststart master mix (Roche) 10µl
- Rox dye (6µM) (Roche) 1.5µl
- Forward Primer (20µM) 0.2µl
- Reverse Primer (20µM) 0.2µl
- Probe (10µM) 0.1µl
- 18S (0.08µM probe 0.02µM primer) 0.3µl
- H_20 5.7µl
- cDNA. 2µl

2.5.3 Analysis of results

Relative quantification was performed using the ABI Prism sequence detection system. Real-time PCR reactions are characterised by the point in time during cycling when amplification of the target reaches a threshold rather than by the amount of target which has accumulated by the end of the reaction. The earlier that a target becomes detectable therefore means the more cDNA present. The results of the TaqMan reaction are presented in an amplification plot, displaying the amount of reporter dye generated during amplification and relative to the quantity of PCR product formed, which is related to the levels of expression of the target gene. The threshold level is defined as the point at which an increase in signal is associated with an exponential increase in PCR product. The threshold cycle (Ct) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold (Figure 2-4). The FAM Ct value corresponds to the cycle number where the fluorescence generated crosses the threshold level. Samples are normalised against the amount of DNA present for a stably expressed 'housekeeping' gene, such as 18S. The 18S ribosomal RNA is a measure of the total RNA content in the sample, and allows for sample variation.

The results were analysed using the comparative ct method, whereby the fold change in gene expression is made relative to a reference sample (e.g control). The initial step in the analysis was to calculate the ΔCt, this is the difference between FAM Ct and 18S Ct. The mean ΔCt for the triplicates was calculated and used to determine the ΔΔCt. The ΔΔCt is the difference between the ΔCt of the experimental sample compared to that of the control sample. The amount of amplified target is given the value $2^{-\Delta\Delta Ct}$, which is based on the mathematical equation that describes the exponential amplification of a PCR:

$$Xn=X_0 \times (1+E_x)^n$$

Where Xn=number of target molecules at cycle n of the reaction, where X_0 is the number of target molecules, E_x is the efficiency of target amplification and n is the number of cycles.

The selected reference or control sample is given the value $2^{-\Delta\Delta Ct}$ of 1

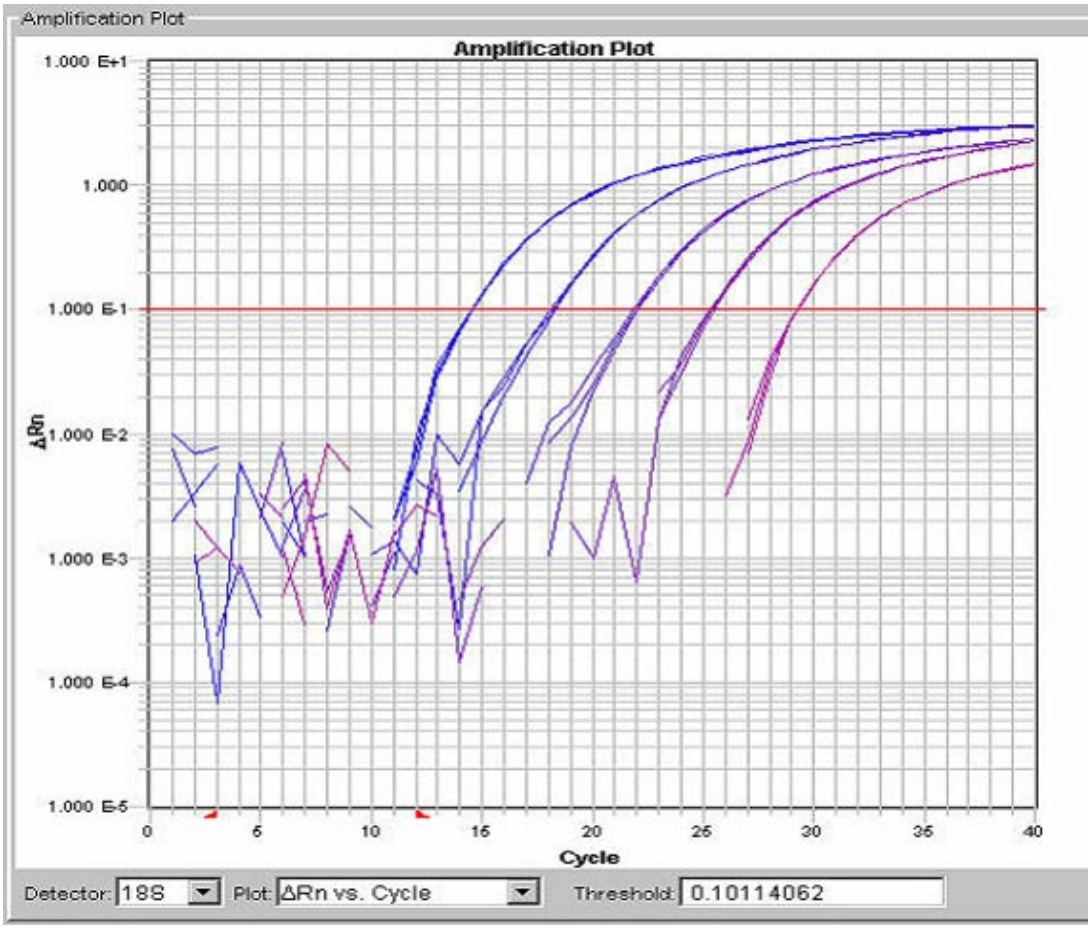

Figure 2-4 A typical amplification plot, after a successful PCR reaction

2.5.4 Primer/Probe validation

The amplification efficiencies of the target and reference must be approximately equal for the $2^{-\Delta\Delta Ct}$ calculation to be valid. In order to asses whether two amplicons have the same efficiency, it should be determined how ΔCt varies with template dilution. An example is shown in Figure 2-5, where a series of RNA dilutions were amplified using primers and probes for SOX9 and 18S. The average Ct was calculated for both SOX9 and 18S, and the ΔCt determined. A plot of log RNA

dilution versus ΔCt was then made. If the absolute value of the slope is close to zero (in the case of SOX9 it was -0.0166), the efficiencies of the target and reference are similar and the ΔΔCt calculation can therfore be used for relative quantification.

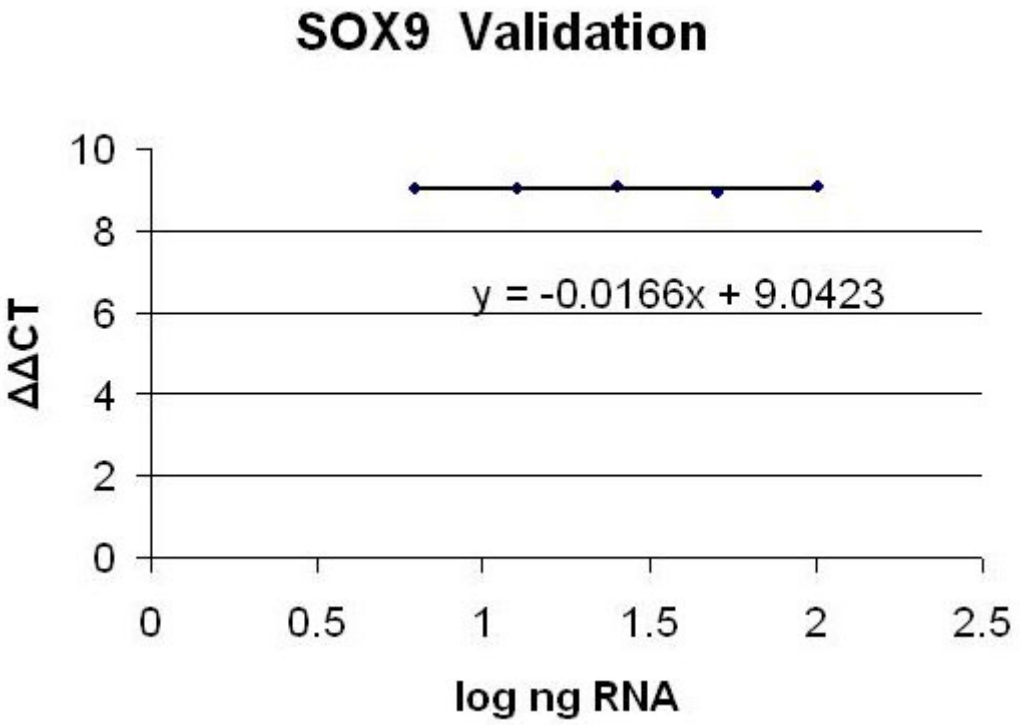

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Figure 2-5 SOX9 validation with log ng RNA plotted against ΔΔCT.

2.6 Western Blotting

Western blotting is a technique where electrophoresis is used to separate proteins by size, the proteins are then transferred onto a membrane, allowing for detection of proteins of interest using specific primary antibodies. Visualisation is performed with secondary antibodies combined with either chemical or fluorescent detection systems. The LiCor system (LiCor Biosciences, Lincoln Nebraska) is a two colour detection system, whereby two fluorescently labelled secondary antibodies can be detected simultaneously with a fluorescent filter.

2.6.1 Protein extraction from tissues and cells

Protein was extracted from human fetal testes and ovaries of the 1st and 2nd trimester. Testes and ovaries from the 1st trimester were placed in 50µl of RIPA Buffer (section

2.7) containing a protease inhibitor cocktail (Roche). 2nd trimester testis were halved and placed in 200µl of RIPA buffer. They were homogenised using a rotorstator homogenizer or with a Qiagen Tissue Lyser. Cells were lysed directly in RIPA buffer. Homogenates were incubated for 1 hour on ice and then centrifuged for 20 minutes at 2500 rpm, the supernatant was kept and the pellet discarded.

2.6.2 Protein quantification

A Biorad DC Protein Assay Kit (Hemel Hempstead, Herts, UK) was used for quantification of proteins, by comparing the amount of protein in diluted samples to a series of controls containing a known concentration of protein Bovine Serum Albumin (BSA) (0.125mg/ml-1.5mg/ml). The standards and samples of an unknown concentration were subjected to an assay based on the Lowry method for protein quantification. The first step involves a reaction between the protein and copper in an alkaline medium, while the second step involves the reduction of Folin reagent by the copper treated protein. The protein causes a reduction of the follin reagent and results in the production of a reduced species with a characteristic blue colour. Briefly, in accordance with manufacturer's instructions, 25µl of reagent S was added to each ml of reagent A. The assay was performed in a flat bottom 96 well plate. To each well, 5µl of sample/standard and 25µl of reagent A (+S) and 200µl of reagent B in duplicate for each well. This was mixed and incubated at room temperature for 15 minutes. Samples were then read on a spectrophotometer (Labsystems Miltiskan Ex, VWR) at 690nm. A standard curve from the solution of known concentration was drawn to obtain the equation of the line. This was used to calculate the value of protein in each sample.

2.6.3 Poly-acrylamide gel electrophoresis

Protein samples were separated using NuPage® Novex Bis-Tris ready made polyacrylamide gels (4-12%) (Invitrogen, paisley, UK) using NuPage® MOPS SDS running buffer (Invitrogen). Protein samples were ran on a NuPage® Novex 4-12% Bis-Tris readymade polyacrylamide gel (Invitrogen, paisley, UK) with 1X MOPS

running buffer (Invitrogen). 20µg of protein was loaded onto each well on the gel, samples were diluted to this concentration in PBS, 5µl of bromophenol loading buffer and 2µl of reducing agent (both Invitrogen) were added to the protein samples prior to loading. The samples were heat denatured for 5 minutes at 70^{o}C. Protein was kept on ice before loading onto gel; 7µl of SeeBlue plus-2 pre-stained multicoloured molecular weight marker (Invitrogen) was loaded, in a parallel lane on each gel. This marker runs down the gel, along with protein and displays bands of a known molecular weight, allowing the size of the samples to be identified. The gel was run at 120 volts for 2 hours. Electrophoresis was stopped when the bromophenol blue dye reached the bottom of the gel.

2.6.4 Protein transfer

The protein from the gel was transferred onto a PVDF Immobin FL membrane (Millipore UK, Watford, UK), using an electrophoresis unit (Hoefer Scientific Instruments) with 1X transfer buffer (Invitrogen), supplemented with methanol, in accordance with manufacturer's instructions. The electrophoresis unit was set up to contain 2 porous pads, 6 pieces of Whatman paper, all soaked in 1x NuPage Transfer Buffer (Invitrogen). The nitrocellulose membrane (Immobilin P, Millipore, Bedford, UK) was rehydrated in methanol for 30 seconds and then washed in transfer buffer. The transfer tank was set up with the membrane on the positive side of the electrode, the arrangement is shown in Figure 2-6. The tank was filled with the above transfer buffer and the blot was transferred at 40 volts for 4 hours or at 20 volts overnight. During this time proteins migrated out of the gel towards the positive electrode and became trapped in the membrane.

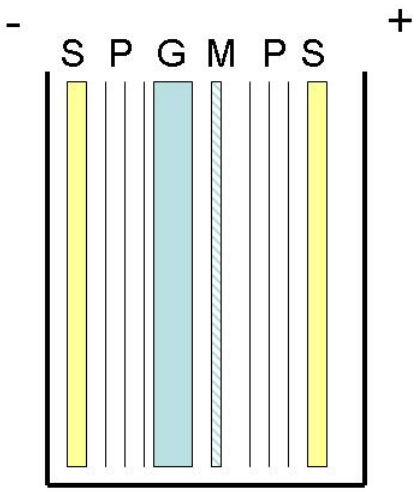


Figure 2-6 Orientation of a Western blot transfer tank. From the negative electrode there is a sponge, followed by 3 pieces of Whatmann paper (P), the gel (G), the membrane (M), 3 pieces of Whatman (P), a sponge (S) and then a positive electrode

2.6.5 Probing the membrane

After transfer to the membrane, non-specific binding was blocked by incubation for 1 hour in Odyssey blocking buffer (LiCor), diluted 1:1 in PBS. Primary antibodies were incubated overnight at 4^{o}C. Details of antibodies used, their concentration, species and source can be found in Tables 3-1, 6-2, 5-5.

After 4x5 minute washes in PBS 0.1% Tween (Sigma), secondary antibodies were added, diluted 1 in 10,000 in Odyssey blocking buffer. The secondary antibodies used are described in Table 2-2. Secondary antibodies were incubated for 1 hour.

Table 2-2 Summary of secondary antibodies used for Western blotting

Antibody	Source	Detection
Goat anti-rabbit	Rockland, USA	800nm
Goat anti-mouse	Molecular Probes, Leiden, The Netherlands	680nm
Donkey anti-goat	Invitrogen, Paisley, UK	680nm

2.6.6 Protein expression analysis

Finally, 4x5 minute washes in PBS 0.1% Tween were performed, the membrane was washed further in PBS to remove any residual Tween 20, and visualised using the LiCor system.

2.7 Commonly used solutions

RIPA lysis Buffer

- 25mM tris
- 1% triton
- 0.05% sodium deoxycholate
- 0.1% SDS
- 150mM NaCl

Tris Buffered Saline (TBS)

- 60.5g Tris
- 87.6g NaCl
- 300mls HCL
- pH was adjusted to 7.4 using HCl

Tris-Acetate EDTA buffer

- 242g Tris base
- 57.1ml Acetic acid
- 100ml 0.5M EDTA

Add ddH_2O to 1 liter and adjust pH to 8.5.

Modified Davidsons fixative

- 37-40% Formaldehyde
- 15% Ethanol
- 5% Glacial Acetic Acid
- 50% water

Media

Embryonic stem cell medium

- 340 mls UHP H_2O
- 40mls of 10x Glasgow Minimal Essential Media
- 13.2mls of Sodium Bicarbonate (7.5% stock solution)
- 4mls of non essential amino acids
- 8mls of glutamine/pyruvate
- 400µl mercaptoethanol
- 40ml foetal calf serum

TVP

- 2.5 ml trypsin
- 0.078g EDTA
- 2.5ml Chick serum
- 245ml PBS

Glutamine/pyruvate

- Sodium pyruvate 100mM
- L-glutamine 200mM

2-mercaptoethanol

- 100µl of mercaptoethanol in 14.1ml sterile water

Human fetal testis somatic cell medium

- Dulbecco's Modified Eagles Medium

Supplemented with:

- 10% HI foetal calf serum
- 1% non essential amino acids
- 1% D-(+)-Glucose (45% solution)
- 1% 2mM L-Glutamine
- 1% Penicillin-Streptomycin
- 1% Fungizone

T9 retinoic acid reporter cells medium

- Dulbecco's Modified Eagles Medium

Supplemented with:

- 20% charcoal stripped foetal calf serum
- 1% non essential amino acids
- 1% 2mM L-Glutamine
- 1% Penicillin-Streptomycin

3 Protein expression patterns in germ cells of the human fetal testis

3.1 Introduction

Primordial germ cells (PGCs) arise from a population of cells within the proximal epiblast (Lawson and Hage, 1994) they migrate to the genital ridge (Kaufman, 1992), arriving by the 5th week of gestation in the human (Wartenberg, 1981) (Chapter 1, section 1.3.1.7). In the male, upon entry to the developing gonads, the germ cells become enclosed by the developing Sertoli cells thus forming the testicular cords, this is complete by the 8th week of gestation (Wartenberg, 1981).

Early germ cells express a number of characteristic pluripotency markers, such as the transcription factors OCT4 and NANOG. In the rodent, both *Oct4* and *Nanog* have been shown to be expressed in PGCs throughout the migratory period and in the initial gonocyte population that colonise the genital ridge (Rosner et al., 1990; Yamaguchi et al., 2005). The germ cells of the rodent then differentiate in a synchronized manner, with all germ cells switching on mouse vasa homologue *(Mvh)* upon entry to the genital ridge (Toyooka et al., 2000), followed by the subsequent downregulation of *Oct4* before birth (Ferrara et al., 2006; Zayed et al., 2007). Studies in the 1970s suggested that unlike the mouse, germ cells of the human fetal testis appear as a heterogenous population of cells within one single testis cord (Fukuda et al., 1975; Wartenberg, 1976). Fukuda et al (1975) used histological analysis to identify 3 different subpopulations of human fetal germ cells, which were termed gonocytes, intermediate cells and prespermatogonia (Chapter 1, section 1.3.2.2). The gonocytes have been shown to express OCT4 and the tyrosine kinase receptor KIT, and have low levels of the melanoma associated antigen, MAGE-A4. The prespermatogonia display little to no expression of OCT4 and KIT, but express high levels of MAGE-A4 protein. An intermediate population of cells with low to negative levels of OCT4 and no KIT or MAGE-A4 has also been identified (Gaskell et al., 2004). The gonocytes are the predominant cell type in the 1st trimester testis

and by the 2nd trimester, gonocytes, intermediate cells and prespermatogonia are all present, with the number of prespermatogonia increasing towards the end of the 2nd trimester.

Understanding germ cell differentiation in the human fetal testis may be key to understanding mechanisms leading to testicular germ cell tumours (TGCT) in adults (Chapter 1, section 1.3.2.3). TGCTs arise from a common precursor lesion called carcinoma in situ (CIS). CIS cells bear close morphological similarity with human fetal germ cells (Nielsen et al., 1974) and it is widely speculated that CIS cells arise from a failure of fetal germ cells to undergo the normal maturation process, instead they are believed to persist in the testis and undergo malignant transformation post-pubertally (Skakkebaek, 1972). This theory is supported by the fact that CIS cells express a large number of proteins in common with embryonic germ cells. Among the proteins detected in both fetal germ cells and CIS are the pluripotency markers OCT4 (Looijenga et al., 2003) and NANOG (Hart et al., 2005), as well as mature markers such as: M2A, a glycosylated monomeric sialoglycoprotein, which has an unknown function (Giwercman et al., 1988); the RNA-binding protein DAZL (LitSchitz-Mercer, 2002); the RNA helicase VASA (Honecker et al., 2004; Zeeman et al., 2002) and the transcription factor AP2γ (Hoei-Hansen et al., 2004; Pauls et al., 2005).

A number of genes have been implicated in the development and maturation of germ cells. One such gene is *DAZL*, a member of the deleted in azoospermia (DAZ) family of proteins that has two further family members DAZ and BOULE, both of which are expressed in the adult human testis (Xu et al., 2001). *Dazl* knock-out mice are infertile (Ruggiu et al., 1997). In female mice, germ cells were lost at e17.5 having failed to complete meiotic prophase, while in males germ cells were reported to be lost as they progressed through the zygotene stage during the first spermatogenic wave, at the time of puberty. Taken together these data suggested that Dazl is important for progression through meiotic prophase in both male and female germ cells (Saunders et al., 2003). Studies in *Dazl*−/− mice of C57BL/6 background

suggested that *Dazl* may have an earlier role, as germ cells in these mice were lost during embryonic development, between e12.5 and e13.5 (Lin and Page, 2005).

Another gene with a well characterized role in germ cell development in the mouse is *Mvh*, a member of the DEAD-box protein family, initially identified as a key regulator of germ cell development in Drosophila (Lasko and Ashburner, 1988). *Mvh* was reported to be germ cell specific (Fujiwara et al., 1994) and Toyooka et al (2000) showed that Mvh was expressed by both male and female germ cells immediately after colonization of the genital ridge through to post-meiotic stages. Loss of *Mvh* resulted in germ cells in the male failing to pass through the zygotene stages of meiosis (Tanaka et al., 2000). In the human, VASA has been shown to be expressed in fetal germ cells of the testis where it localized to OCT4 negative cells of a 21 week of fetus (Honecker et al., 2004).

Another RNA binding protein, nanos was originally identified as a maternal effect gene in Drosophila (Wang and Lehmann, 1991), where it is necessary for the migration and development of germ cells in Drosophila embryos (Forbes and Lehmann, 1998; Kobayashi et al., 1996), mediating its effects through interaction with the pumilio protein (Abe et al., 1996; Forbes and Lehmann, 1998). In the mouse the *Nanos* gene has been cloned, as have 2 homologues (*Nanos 2 and 3*) (Tsuda et al., 2003). In the human NANOS1 may have a role in germ cell development, as it has been shown to bind to human PUMILIO2, both of which are co-expressed in spermatogonia (Jaruzelska et al., 2003).

3.1.1 Aims of chapter

The aim of this study was to further characterize germ cells in the human fetal testis, by studying their pattern of protein expression during the 1st and 2nd trimester, using double fluorescent immunohistochemistry to determine protein expression in specific germ cell types and Western blotting to compare total protein expression.

3.2 Materials and methods

3.2.1 Collection of human fetal testes

Human fetal testes were obtained following termination of pregnancy as outlined in section 2.1

3.2.2 Testicular germ cell tumour tissue

Testicular germ cell tumour samples were collected by the pathology departments at Edinburgh Royal Infirmary and Edinburgh Western General. Ethical approval was obtained from Lothian Local Research Ethics Committee (REC ref:07/51101/8).

3.2.3 Western blotting

Western blotting was performed on human fetal testes of the 1st trimester and 2nd trimester. Testes were halved and lysed in RIPA buffer (section 2.6.1). Protein was quantified using a Biorad protein assay kit (section 2.6.2). Details of Western blotting are outlined in section 2.6. Information regarding the primary antibodies used is listed in Table 3-1. Quantification of Western blots was performed using the Odyssey software, where the values of the proteins of interest were divided by the values for the loading control. Values were then made relative to one of the 9.3 day fetal testis. Statistical analysis was performed using a student's t-test.

Table 3-1 Summary of primary antibodies used for Western blotting

Antigen	Host Species	Source	Dilution
AP2γ	Mouse	Santa Cruz	1.200
DAZL	Mouse	Serotec	1.500
NANOG	Goat	R and D systems	1.500
NANOS1	Rabbit	AbCam	1.500
OCT4	Goat	Santa Cruz	1.500
VASA	Rabbit	AbCam	1.500
β-TUBULIN	Rabbit	Santa Cruz	1.1000
β-TUBULIN	Mouse	Sigma	1.1000

3.2.4 Haematoxylin and eosin staining

Details of the haematoxylin and eosin procedure are outlined in section 2.3.1.

3.2.5 Immunohistochemistry

3.2.5.1 DAB

The immunohistochemical procedure used is outlined in section 2.3.2. Information regarding the antibodies used is listed in Table 3-2.

Table 3-2 Summary of primary antibodies used for immunohistochemistry

Antigen	Source	Species	Dilution	Citrate Retrieval
AP2γ	Santa Cruz	Mouse	1.20	Yes
DAZL	Serotec	Mouse	1.300	Yes
Ki67	AbCam	Rabbit	1.200	Yes
Ki67	Vector	Mouse	1.100	Yes
M2A	AbCam	Mouse	2 drops/slide	Yes
NANOG	R+D systems	Goat	1.50	Yes
NANOS1	AbCam	Rabbit	1.500	Yes
OCT4	Santa Cruz	Goat	1.50	Yes
VASA	AbCam	Rabbit	1.500	Yes

3.2.5.2 Immunofluorescence

Immunofluoresent immunohistochemistry was performed as described in section 2.3.10. The protocols for double fluorescent immunohistochemistry using specific primary antibodies are outlined in Table 3.3.

Table 3-3 Summary of primary and secondary antibodies, and fluorescent labels used for co-localisation

Antigen 1	**Dilution**	**Secondary antibody**	**Detection method**	**Antigen 2**	**Dilution**	**Secondary antibody**	**Detection method**
DAZL	1.500	GAM-b	Strep 546	VASA	1.300	GAR -488	-
VASA	1.300	ChAR-b	Strep 488	OCT4	1.200	ChAG-p	Tyr cy 3
AP2γ	1.60	GAM-p	Tyr cy 3	VASA	1.200	GAR-b	Strep 488
VASA	1.200	GAR (Fab)-b	Strep 488	NANOS1	1.60	GAR (Fab)-b	Strep 546
VASA	1.200	GAR-b	Strep 488	OCT4	1.50	ChAG-p	Tyr cy 3
DAZL	1.500	RAM-b	Strep 546	OCT4	1.200	RAG-p	TSA plus fluorescein
VASA	1.300	GAR-b	Strep 488	M2A	2 drops per slide	GAM-p	Tyr cy 3
Ki67	1.100	ChAR-b	Avidin 488	OCT4	1.200	ChAG-p	Tyr cy 3
M2A	2 drops per slide	ChAM-b	Strep 488	OCT4	1.200	ChAG-p	Tyr cy 3
Ki67	1.100	ChAM-b	Strep 488	VASA	1.200	GAR-546	-

ABBREVIATIONS

GAM-b: Goat anti-mouse biotinylated
GAR-488:Goat anti-rabbit alexaflour 488
RAG-p:Rabbit anti-goat peroxidase
ChAR-b:Chicken anti-rabbit biotinylated
RAM-b: Rabbit anti-mouse biotinylated
Strep488:Streptavidin 488
Strep546: Streptavidin 546
ChAM-b:Chicken anti-mouse biotinylated
ChAG-p:Chicken anti-goat peroxidase
GAR-546:Goat anti-rabbit alexafluor 546
GAR (fab)-b:Goat anti-rabbit FAB-biotinylated
TyrCy3: Tyramide Cy3
TSA (tyramide signal amplification)

3.2.6 Proliferation index

Ki67 immunohistochemistry was used to identify proliferating germ cells. Fetal testes were divided into groups of early 2nd trimester (14-15 weeks, N=4), mid 2nd trimester (16-17 weeks, N=5), and late 2nd trimester (18-19 weeks, N=4). Fetal germ cells were identified based on their morphology and location within the

testicular cords. Counting was performed using a 40x objective fitted to an Olympus BH2 microscope with Hitachi HVC20 camera and images were analysed using Imagepro-plus version 4.5.1 with Stereology Pro 5.0 (Media Cybernetics, Wokingham, Berkshire, UK); 30 fields were randomly selected and counted. The proliferation index was calculated by dividing the number of Ki67 positive germ cells by the total number of germ cells counted. Statisitical analysis was performed using a one-way analysis of variance (ANOVA).

3.3 Results

3.3.1 Western analysis of 1st and 2nd trimester human fetal testes

Total protein expression of putative germ cell markers in extracts of testes of the 1st and 2nd trimester were determined using Western blot analysis. The microtubular protein β-tubulin was used as a loading control. OCT4 (39kD), NANOG (37kD), AP2γ (50kD), NANOS1 (35kD) and DAZL (33.5kD) were all detected in both 1st and 2nd trimester samples (Figure 3-1). VASA (76kD) protein was found to be absent in testes of the 1st trimester, but present throughout the 2nd trimester (Figure 3-1).

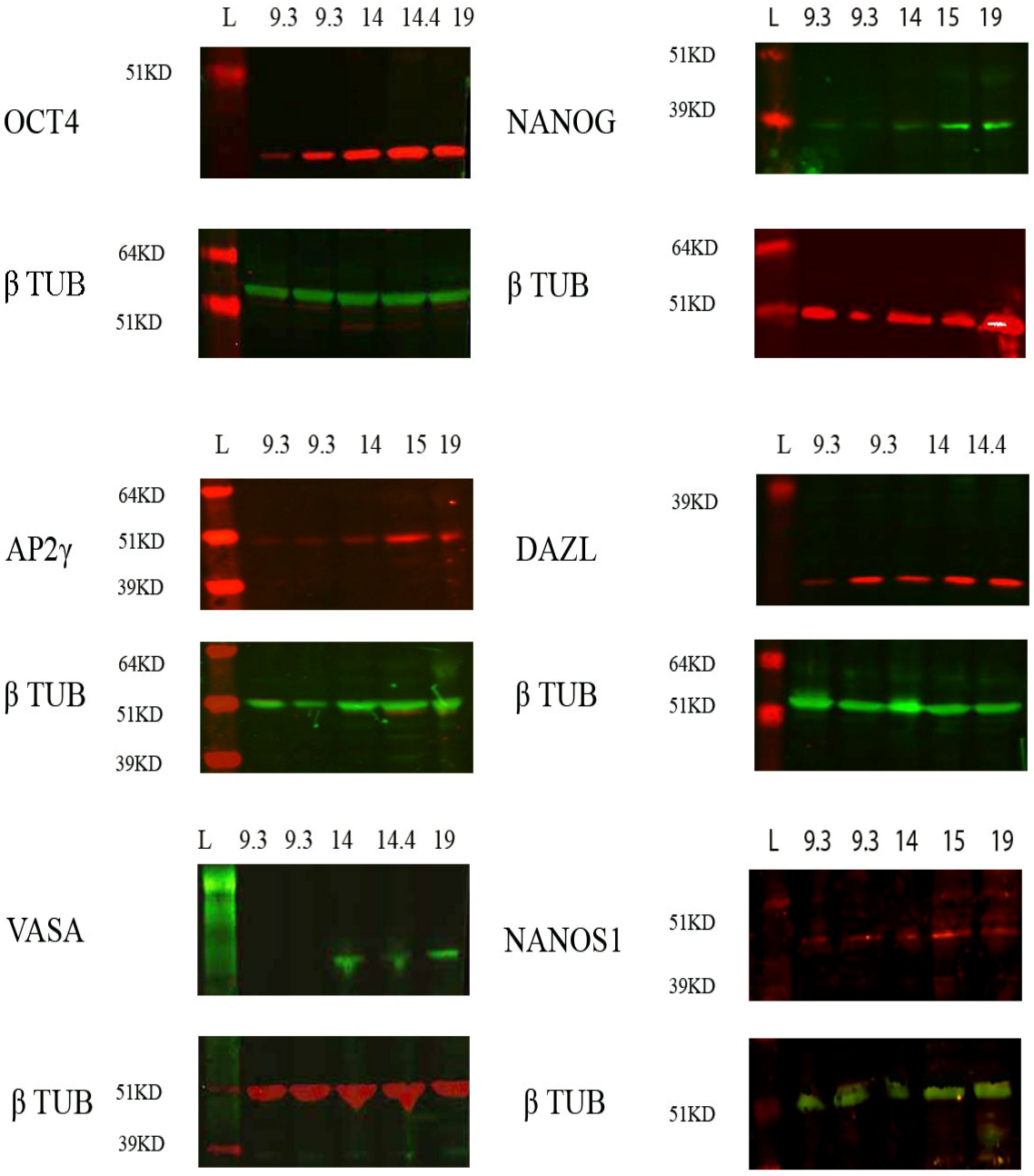


Figure 3-1 Western blots for OCT4, NANOG, AP2γ, DAZL, VASA and NANOS1 in human fetal testes of the 1st (9.3 days) and 2nd trimester (14, 15 and 19 weeks). β-tubulin (51KD) is the loading control for each of the proteins, L is the protein ladder, relative to second trimester testes.

Quantification of proteins revealed that all proteins tended to increase in the 2nd trimester compared with the first (Figure 3-2). The increased expression of these proteins in the 2nd trimester relative to the 1st trimester could be attributed to increased numbers of germ cells expressing these proteins, increased expression per germ cell or increased proportion of germ cells in the testis relative to somatic cells. Therefore to further analyse the expression patterns of these proteins, immunohistochemistry was performed.

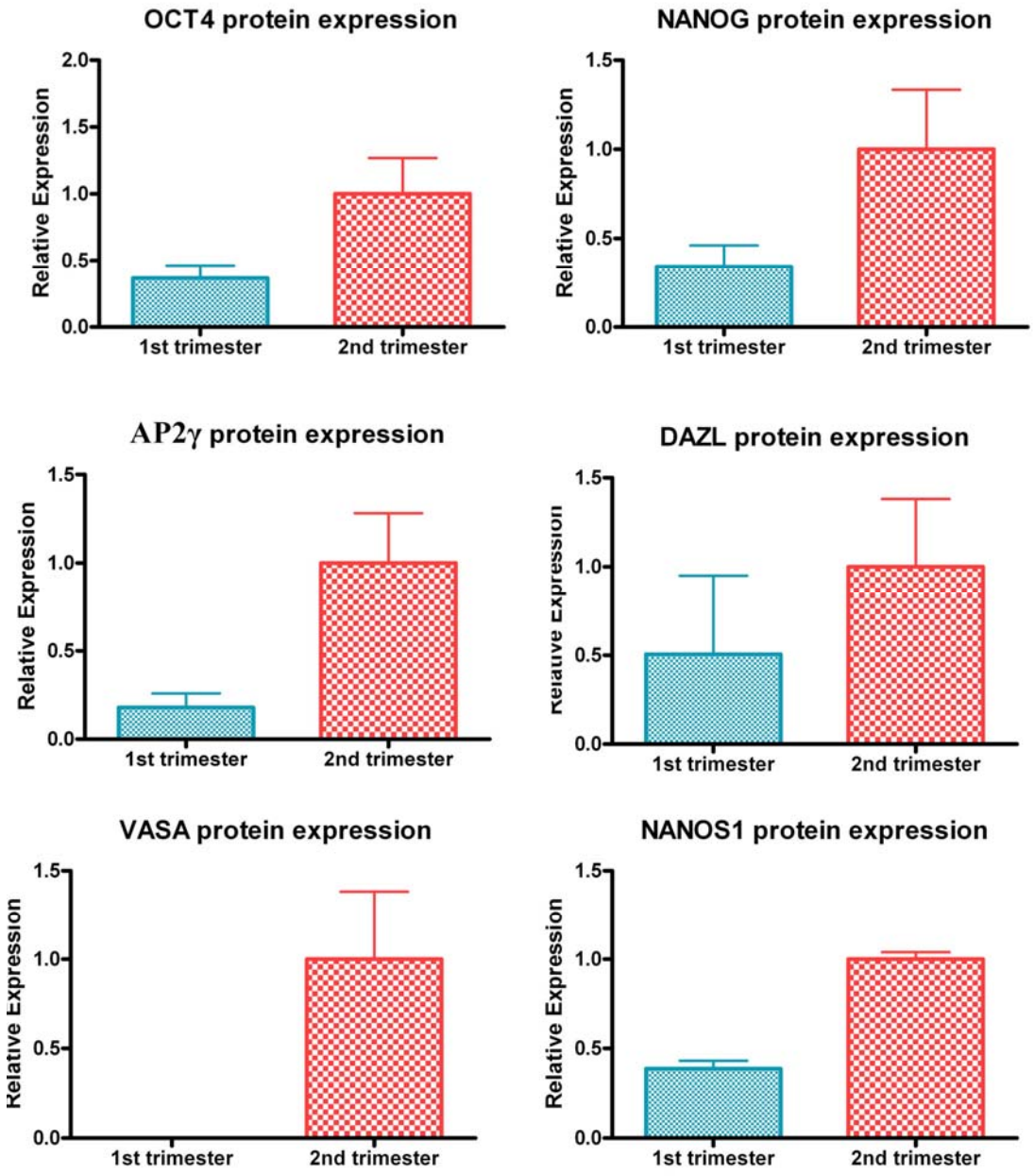


Figure 3-2 Quantification of Western blots OCT4, NANOG, AP2γ, DAZL, VASA and NANOS1 in 1st (N=2) and 2nd (N=3) trimester testes. Mean ± sem.

3.3.2 Immunohistochemical analysis of OCT4, NANOG, AP2γ, and M2A

The expression of OCT4, NANOG, AP2γ and M2A was further investigated using immunohistochemistry. All proteins were expressed in testes of the 1st and 2nd trimester (Figure 3-3 and Figure 3-4). All proteins were localized to the germ cells, identifiable as large round cells with a prominent nucleus. In the 2nd trimester germ cells were located within the testis cords, separated from the interstitium. OCT4, NANOG and AP2γ were restricted to the nuclei of the germ cells. M2A was expressed on the germ cell membrane. All identifiable germ cells were immunopositive for OCT4, NANOG, AP2γ and M2A in the 1st trimester. In testes of the 2nd trimester, a number of negative germ cells could be identified (Figure 3-4, asterisks)

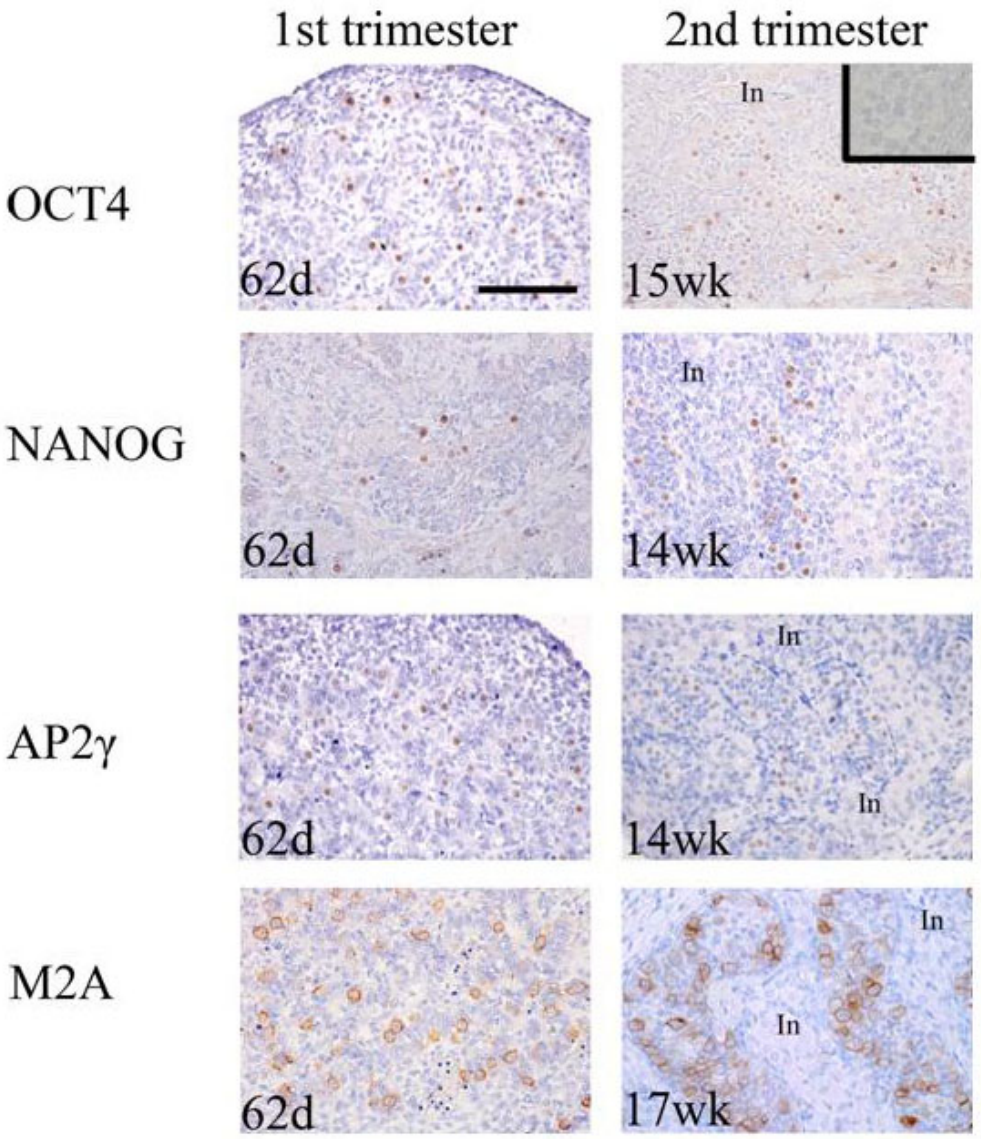


Figure 3-3 Immunohistochemistical staining for OCT4, NANOG, AP2γ, and M2A in testes of the 1st and 2nd trimester (In = Interstitium). Bar =100µm, applies to all images. Inset shows negative control

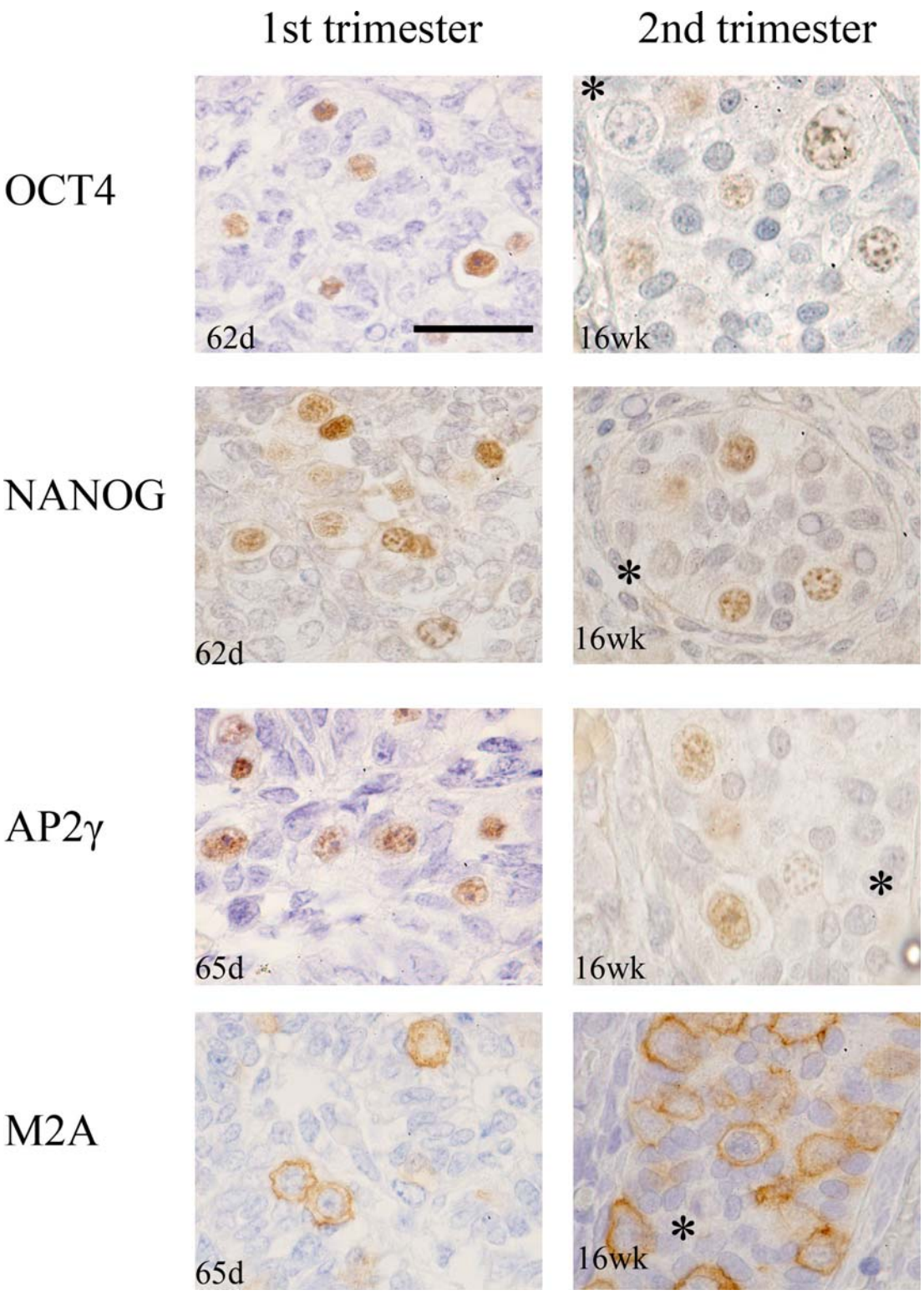


Figure 3-4 High power images of human fetal testes immunohistochemically stained for OCT4, NANOG, AP2γ, and M2A at both the 1st and the 2nd trimester. Bar = 50μm, applies to all images. Asterisks show immunonegative germ cells

3.3.3 Immunohistochemical analysis of DAZL, NANOS1 and VASA

Protein expression of DAZL was demonstrated in testes sections of the 1st and 2nd trimester (Figure 3-5, Figure 3-6). In testes of the 1st trimester, germ cells displayed weak DAZL staining in the nucleus and cytoplasm of germ cells. In the 2nd trimester DAZL staining was more intense and remained in both nuclear and cytoplasmic compartments. NANOS1 staining was found at low intensity in germ cells of the 1st trimester (Figure 3-5, Figure 3-6). In the testis of the 2nd trimester NANOS1 staining was stronger, where it predominantly was found in the nuclear periphery (Figure 3-5). VASA protein expression was not detectable in testis of the 1st trimester, but was found in the cytoplasm of germ cells in 2nd trimester testis (Figure 3-5, Figure 3-6).

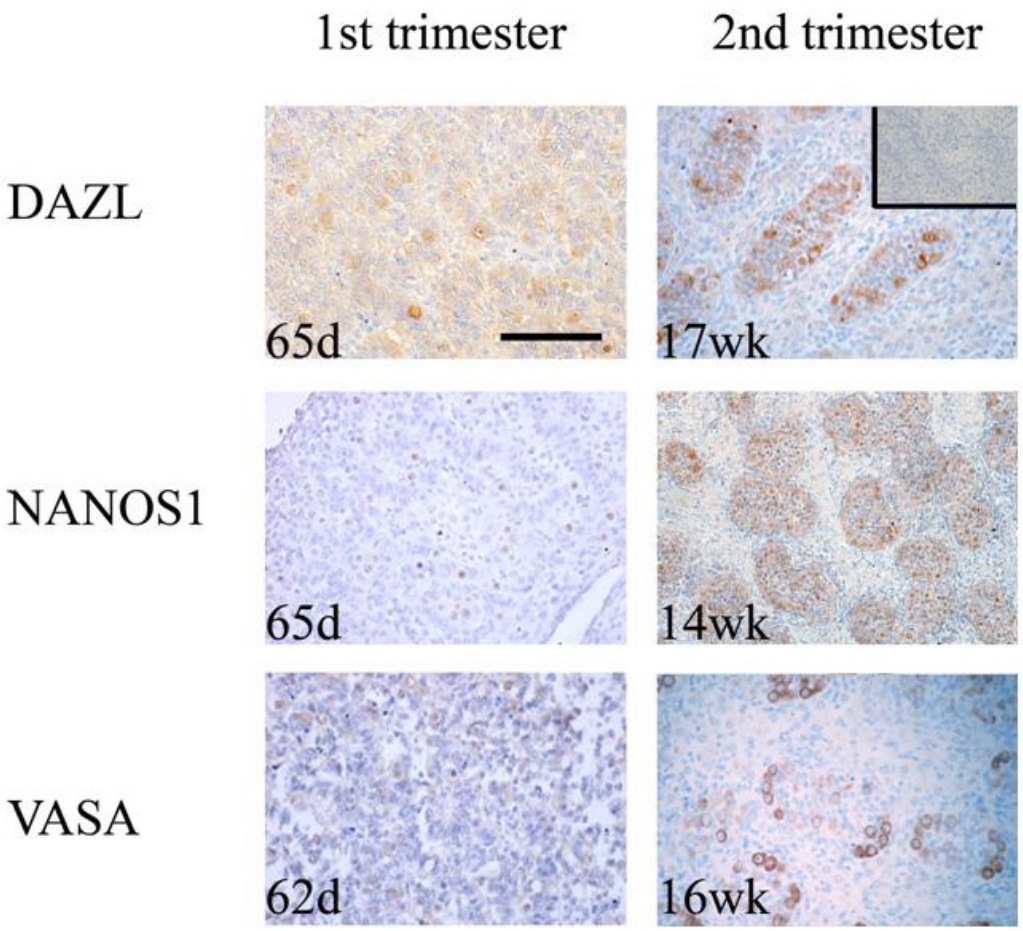


Figure 3-5 Immunohistochemistry for DAZL, NANOS1 and VASA in testes of the 1st and 2nd trimester. Bar=100 µm. Inset shows negative control

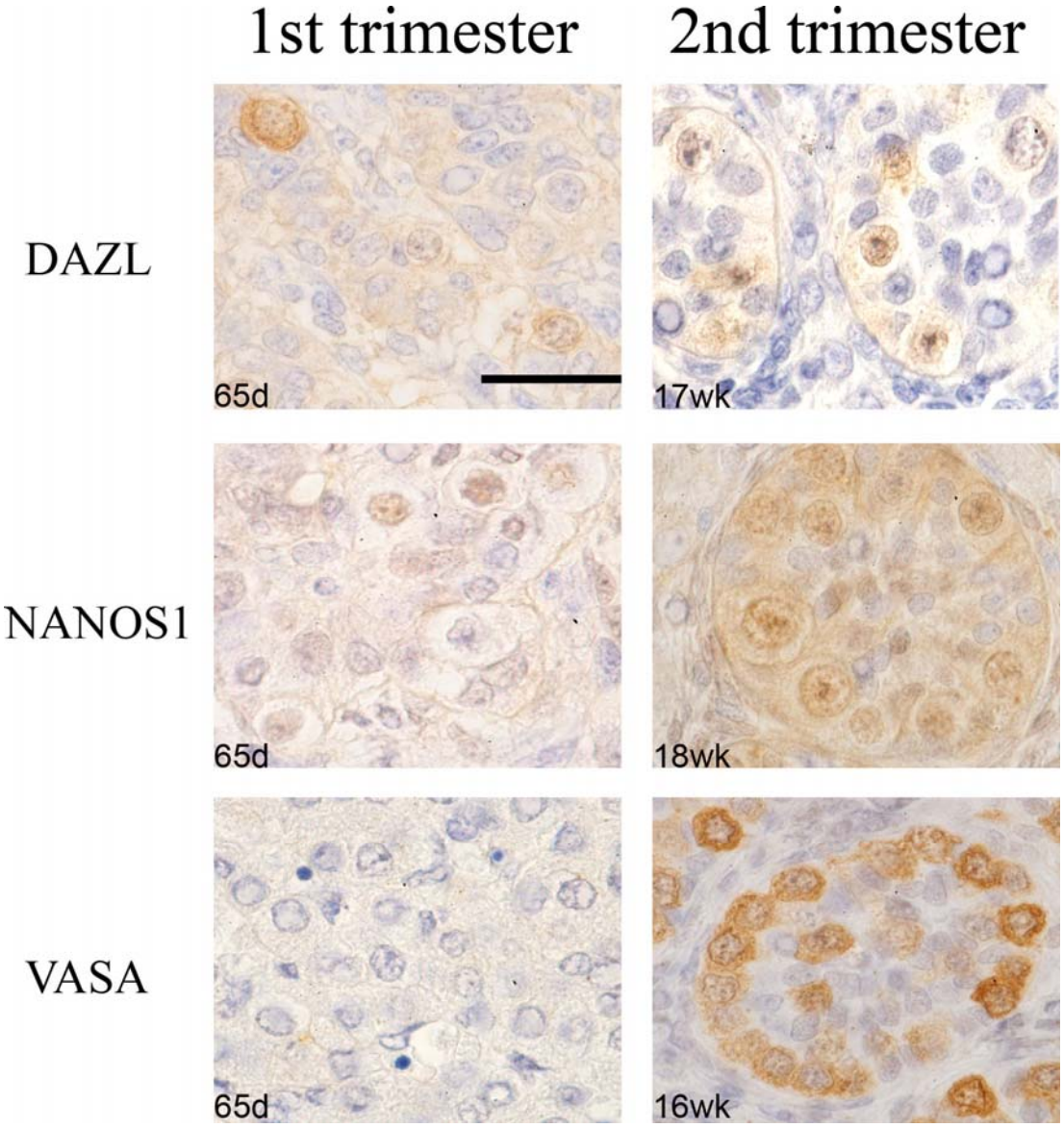


Figure 3-6 High power images of DAZL, VASA and NANOS1 in 1st and 2nd trimester human fetal testes. Bar = 50μm

3.3.4 Immunoexpression of OCT4 and VASA

Immunofluorescent co-staining for OCT4 and VASA was performed on 1st and 2nd trimester testes. In testes of the first trimester all OCT4 positive germ cells were VASA negative. In the 2nd trimester, germ cells with high levels of OCT4 staining tended to have low levels of VASA (Figure 3-7, arrowhead). Conversely cells which stained positive for VASA had low intensity of OCT4 staining (Figure 3-7, arrows). Germ cells could also be identified which displayed low staining of both OCT4 and VASA (Figure 3-7, asterisks), but these were infrequent.

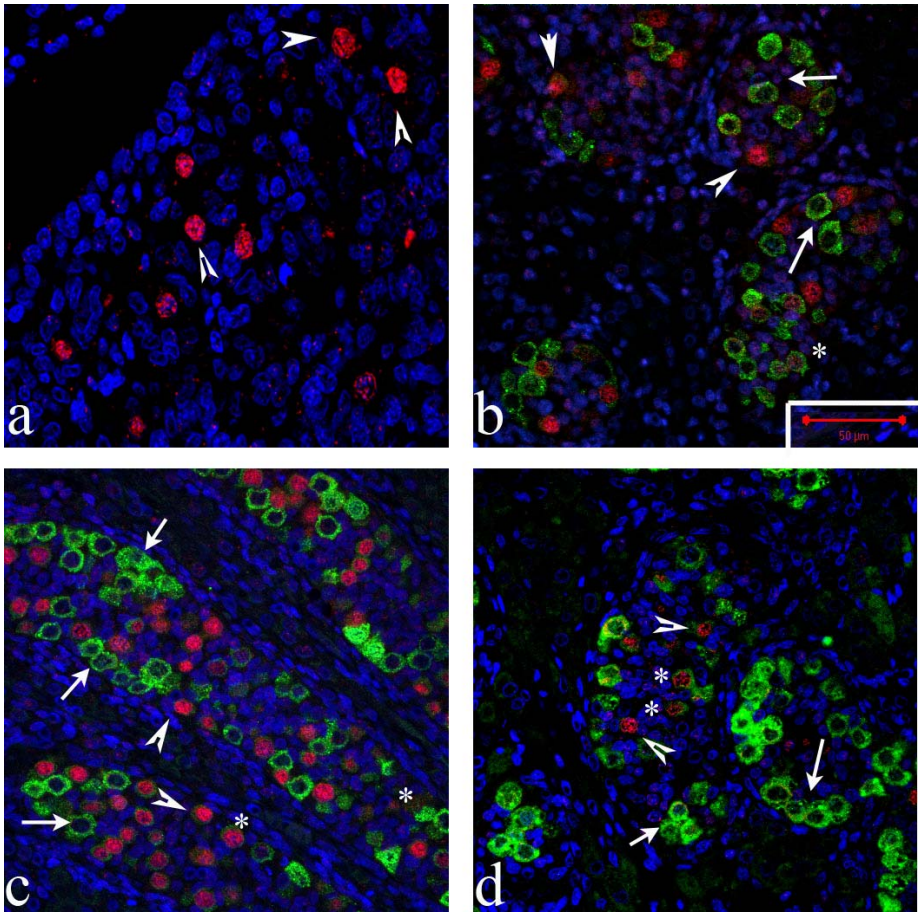


Figure 3-7 Immunofluorescent co-staining for OCT4 (red) and VASA (green) in testes of the 1st trimester a) 65 days and the 2nd trimester b) 14 weeks c) 16 weeks and d)19 weeks. Arrowheads point to OCT4 positive cells, arrows show VASA positive cells. Asterisks show cells with low levels of OCT4 and VASA staining. Bar=50µm

3.3.5 Immunoexpression of AP2γ and VASA

Co-staining for AP2γ and VASA in second trimester testis revealed that AP2γ positive cells were low/negative for VASA, while VASA positive cells were low/negative for AP2γ (Figure 3-8). Arrowheads identify the VASA positive cells, while arrows identify the AP2γ positive cells.

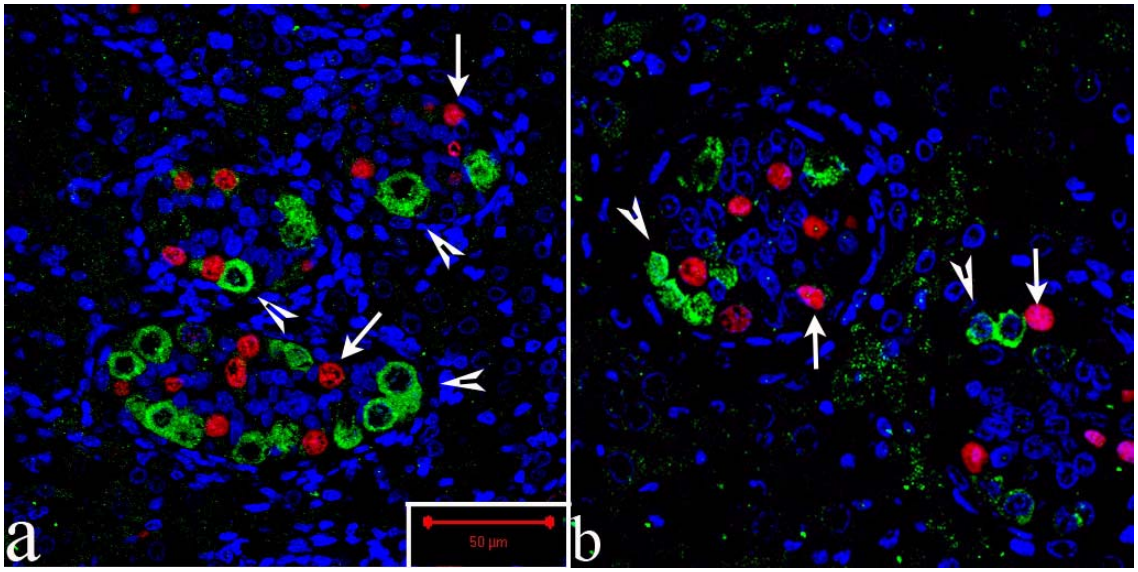


Figure 3-8 Fluorescent immunohistochemistry for AP2γ (red) and VASA (green) in 2nd trimester testes at a) 16 weeks and b) 18 weeks. Arrows point to AP2γ expressing germ cells and arrowheads indicate to germ cells expressing VASA only. Bar=50µm

3.3.6 Immunofluorescent co-staining for OCT4 and M2A

Immunofluorescent co-staining for OCT4 and M2A (Figure 3-9) was performed on 1st and 2nd trimester testis. Both OCT4 and M2A were restricted to the same cell type in both a 65 days and 17 week testis. There was a marked decrease in expression of both proteins by 17 weeks possibly as a result of reduced numbers of germ cells expressing OCT4 and M2A.

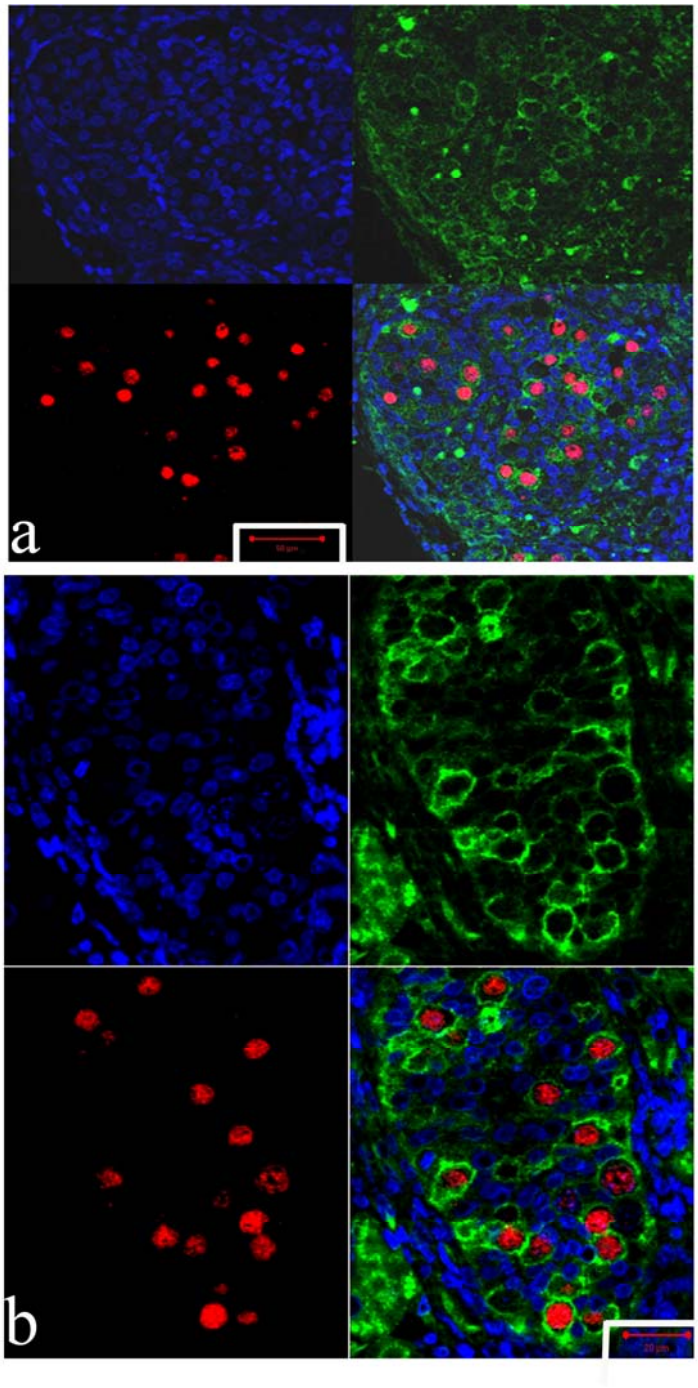


Figure 3-9 Fluorescent immunohistochemical staining for OCT4 (red) and M2A (green) in a) testis of the first trimester at 65 days and b) 2nd trimester at 17 weeks. Cells stained positively for OCT4 also tended to stain positively for M2A. In a) Bar=50 µm and in b) Bar=20 µm

3.3.7 Immunofluorescent staining of M2A and VASA

Immunofluorescent staining for M2A and VASA was performed on 1st and 2nd trimester testis. VASA positive cells had low/negative levels of M2A (arrowheads), while M2A positive cells showed little VASA staining (arrows) (Figure 3-10).

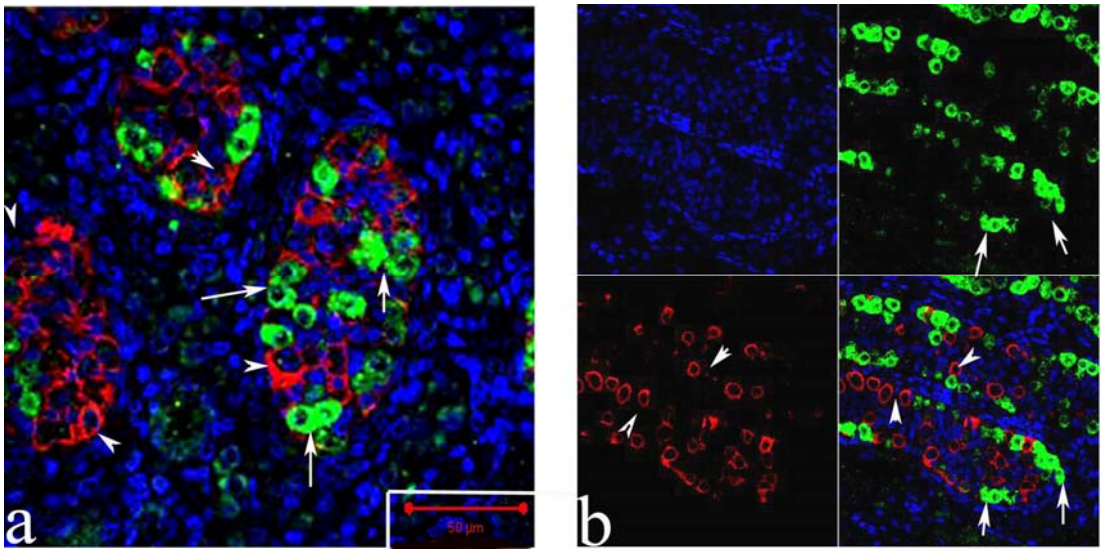


Figure 3-10 Immunohistochemical staining for M2A (red, as indicated by arrowheads) and VASA (green, as indicated by arrows) in testes of the 2nd trimester at a) 17 week and b) 19 week. Bar=50µm

3.3.8 Co-localisation of OCT4 and DAZL

DAZL immunopositive cells were found to be generally restricted to the germ cells which were OCT4 positive. DAZL was immunolocalised to the cytoplasm and nucleus of a number of the OCT4 expressing cells (Figure 3-11).

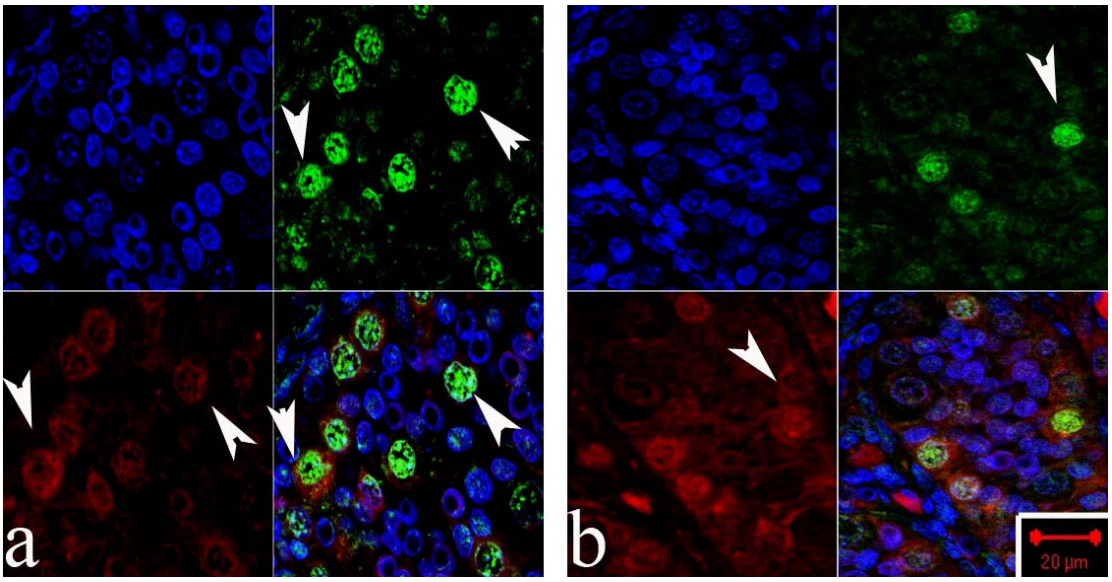


Figure 3-11 Fluorescent immunohistochemistry showing the localisation of OCT4 (green) and DAZL (red) in 2nd trimester testes at a) 15 weeks and b) 19 weeks. Arrowheads show OCT4/DAZL co-localisation. Bar=20 µm

3.3.9 Immunoexpression of DAZL and VASA

Immunofluorescent co-staining for VASA and DAZL revealed that both were generally restricted to separate cell types, although there was some degree of overlap. Cells with the highest levels of VASA staining were generally negative for DAZL. In a number of the DAZL positive/VASA negative cells, DAZL was immunolocalized to the nucleus (Figure 3-12, asterisks).

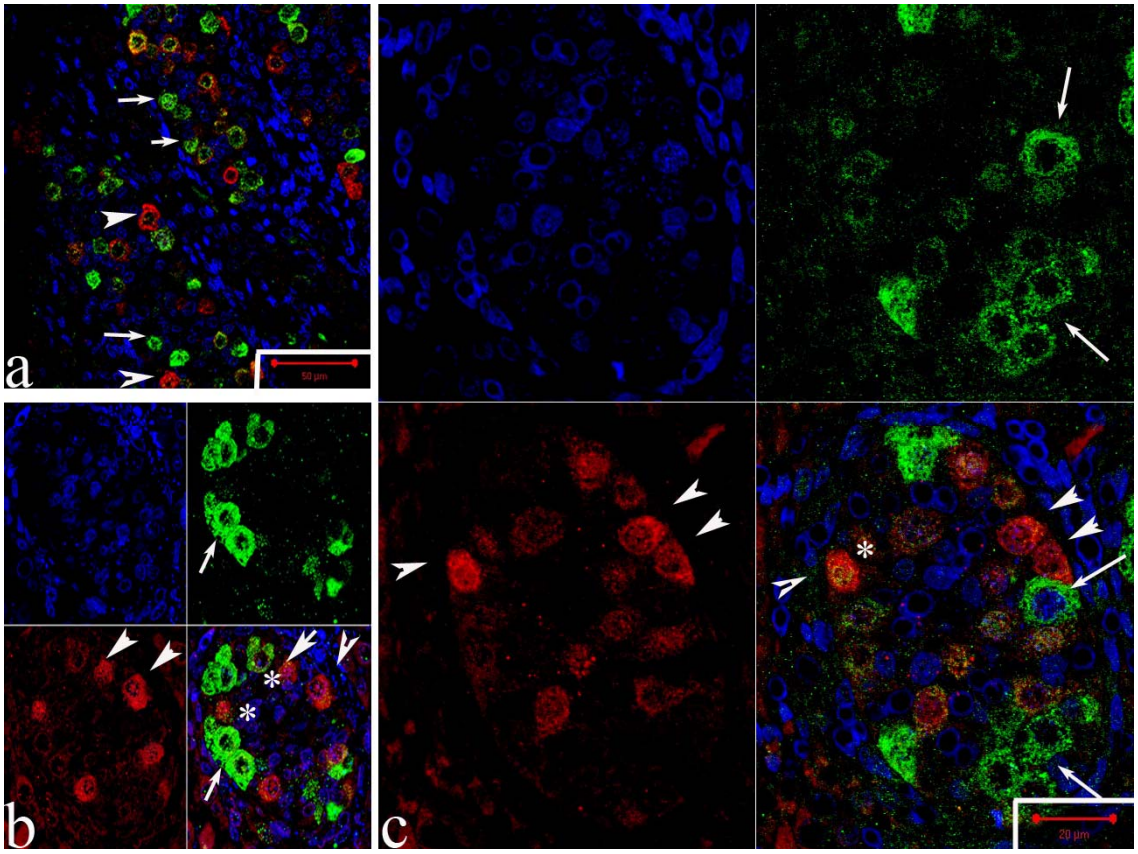


Figure 3-12 Immunofluorescent staining for VASA (green) and DAZL (red) in 2nd trimester testis at a) 15 weeks b) 17 weeks c) 16 weeks. Arrows point to germ cells expressing VASA only. Arrowheads point to DAZL positive/VASA negative cells. Asterisks identify cells with high nuclear DAZL staining. In a) bar=50 µm, in b and c) bar=20 µm.

3.3.10 Immunoexpression of OCT4 and NANOS1 in 2nd trimester human fetal testes

NANOS1 was primarily detected in the nuclei of germ cells (Figure 3-13, arrows), where it was found to be expressed in germ cells which were low for OCT4 (Figure 3-13, arrowheads). Some occasional co-localisation could be seen (Figure 3-13, asterisks).

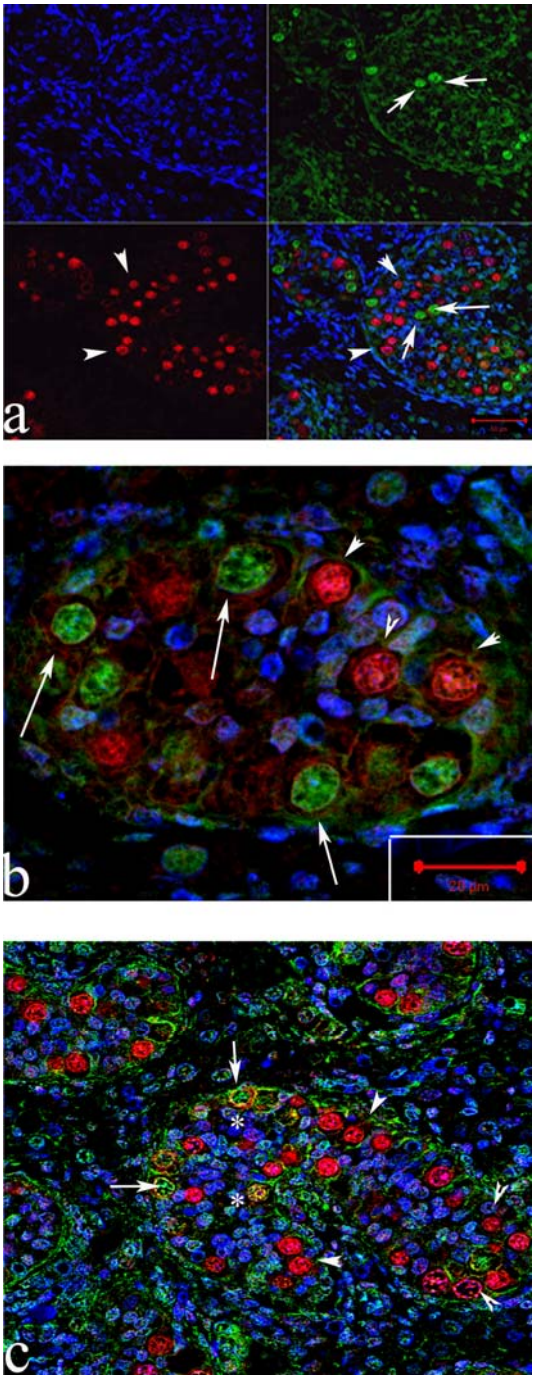


Figure 3-13 Fluorescent immunohistochemistry for NANOS1 (green) and OCT4 (red) in testes of the 2nd trimester a) 16 weeks b) 16 weeks and c) 17 weeks. Arrowheads identify OCT4 positive cells, arrows indicate OCT4 positive cells and asterisks show cells with some co-localisation. In a) bar=50 µm in b) bar=20 µm.

3.3.11 Immunofluorescent co-staining for NANOS1 and VASA

Both NANOS1 and VASA were found to be restricted to the same cells types in germ cells of the 2nd trimester. NANOS1 was found to be immunolocalised to the nuclei of VASA positive germ cells (Figure 3-14).

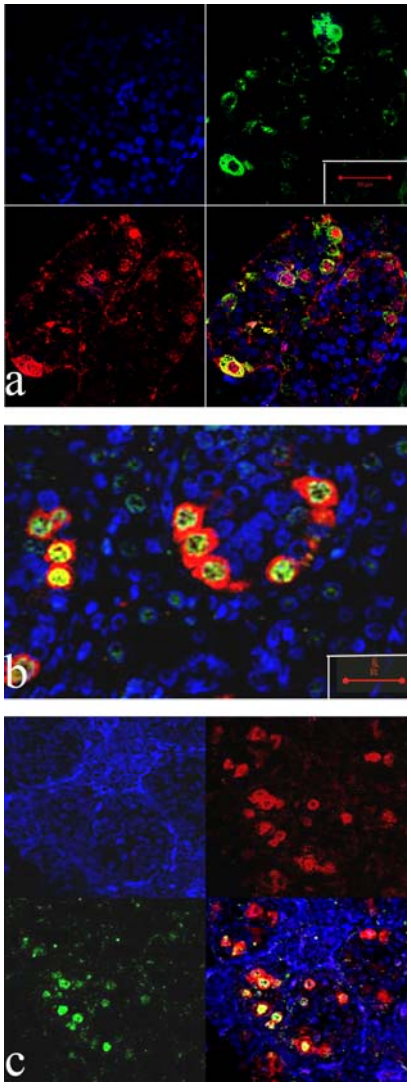


Figure 3-14 Immunoflourescent staining for NANOS1 (green) and VASA (red) in 2nd trimester human fetal testes a) 15 weeks b) 16 weeks c) 18 weeks. Cells which stained positive for NANOS1 also tended to stain positive for VASA. In a bar=50 µm and applies to c also and in b bar=20 µm

3.3.12 Morphological analysis of TGCT samples

TGCT samples were examine for morphological abnormalities and tubules containing CIS were identified, these cells with large prominent nuclei and resembled fetal germ cells (Figure 3-15).

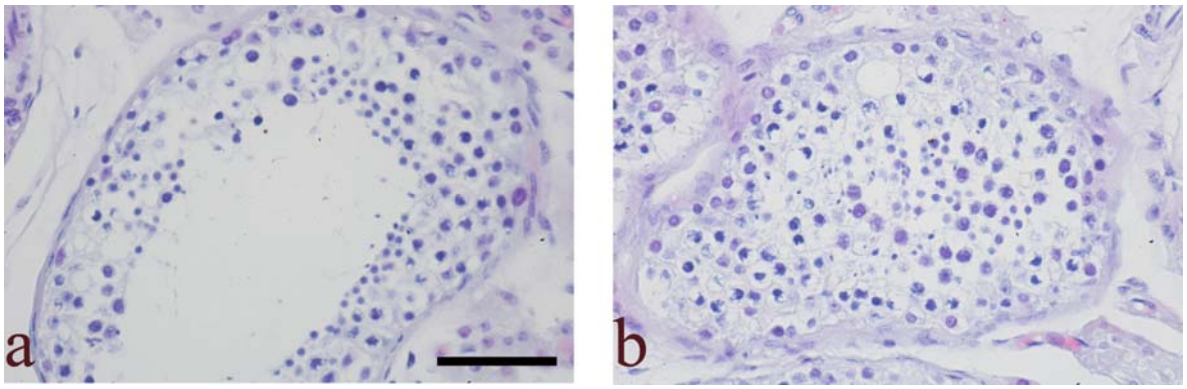


Figure 3-15 a and b) Haematoxylin and eosin staining of TGCT samples containing CIS. Bar=50 µm

3.3.13 Immunofluorescent staining for OCT4 and VASA in TGCT samples

CIS cells were detected within the tubules of TGCT tissue sections (Figure 3-16 a-d). OCT4 positive cells were present but no OCT4 positive cells could be detected in adjacent 'normal' tubules (Figure 3-16 e). Within the tubules containing CIS, VASA immunopositive cells were also present, but these cells did not appear to be immunopositive for OCT4 (Figure3-16 a-d).

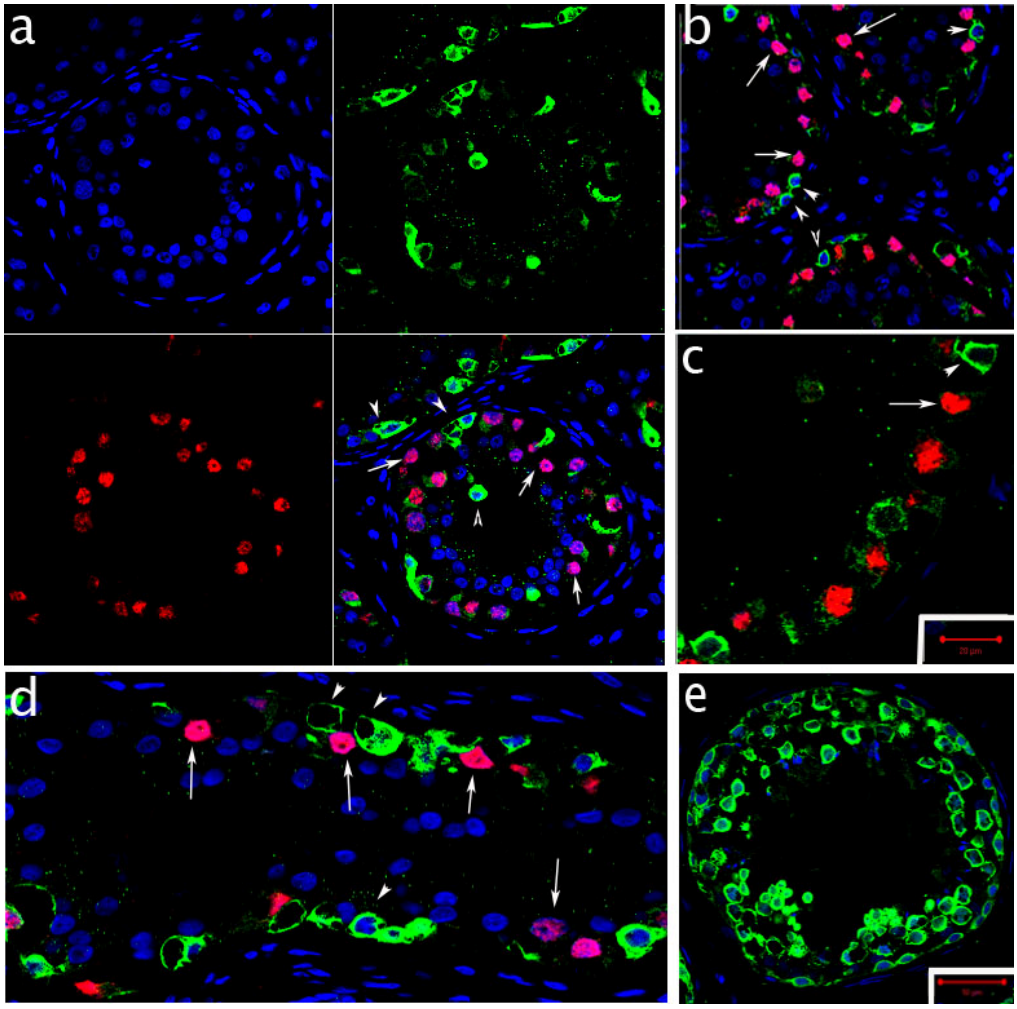


Figure 3-16 Immunofluorescent staining of OCT4 (red) and VASA (green) in adult testicular germ cell tumour samples a) tubule containing CIS cells b) staining of CIS cells from a different TGCT sample c) high power image of b) d) another tissue sample containing CIS and e) a normal tubule containing no CIS. Arrows point to OCT4 positive cells, while arrowheads point to VASA positive cells. In e bar=50 µm and applies to a also, and in c, bar=20 µm and applies to b and d also.

3.3.14 Germ cell proliferation

Ki67 positive germ cells were identified based on their morphology and their location within the cords (Figure 3-17a). Quantification of the number of Ki67 positive germ cells showed that throughout the early and mid 2nd trimester, germ cell proliferation occurred in a relatively high proportion of germ cells, with more than half of the cells proliferating, although the number of proliferating cell tended to decrease by 18-19 weeks, this was non-significant ($P>0.05$) (Figure 3-17b).

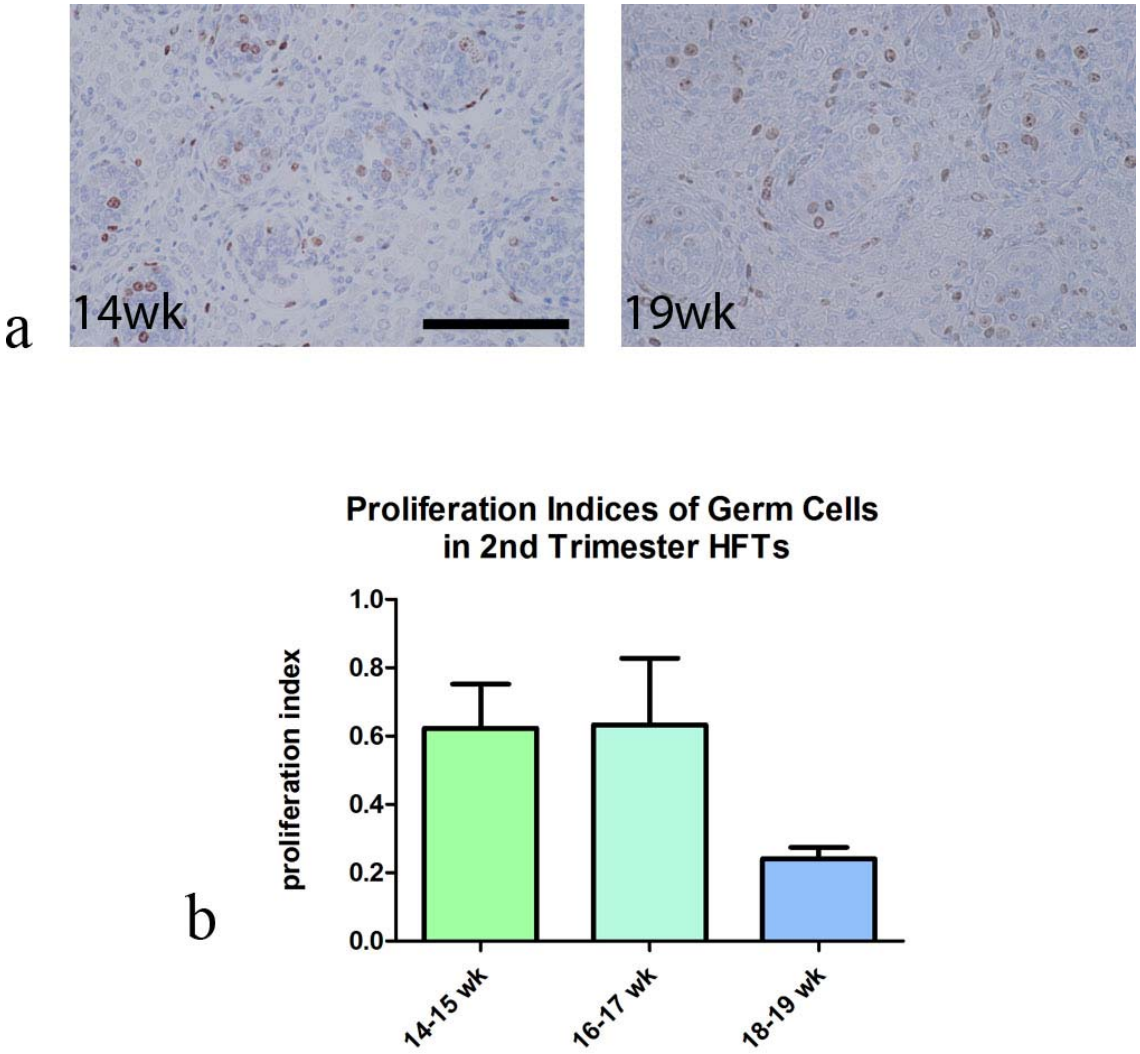


Figure 3-17 Ki67 proliferation in germ cells of the human fetal testis (HFT) a) Immunostaining for Ki67 in 2nd trimester testis. Bar=100µm b) proliferation indices for germ cells in early (14-15 weeks, N=4), mid (16-17 weeks, N=5) and late (18-19 week, N=4) testis. Mean ± sem

3.3.15 Immunoflourescent co-staining of Ki67 with OCT4

Using co-staining for Ki67/OCT4, a high number of germ cells in the 1st trimester were Ki67 positive (Figure 3-18 a and b). In the 2nd trimester some OCT4 immunopositive germ cells were also found to express Ki67 (Figure 3-18 c-d, asterisks), while other cells were positive for OCT4 but negative for Ki67.

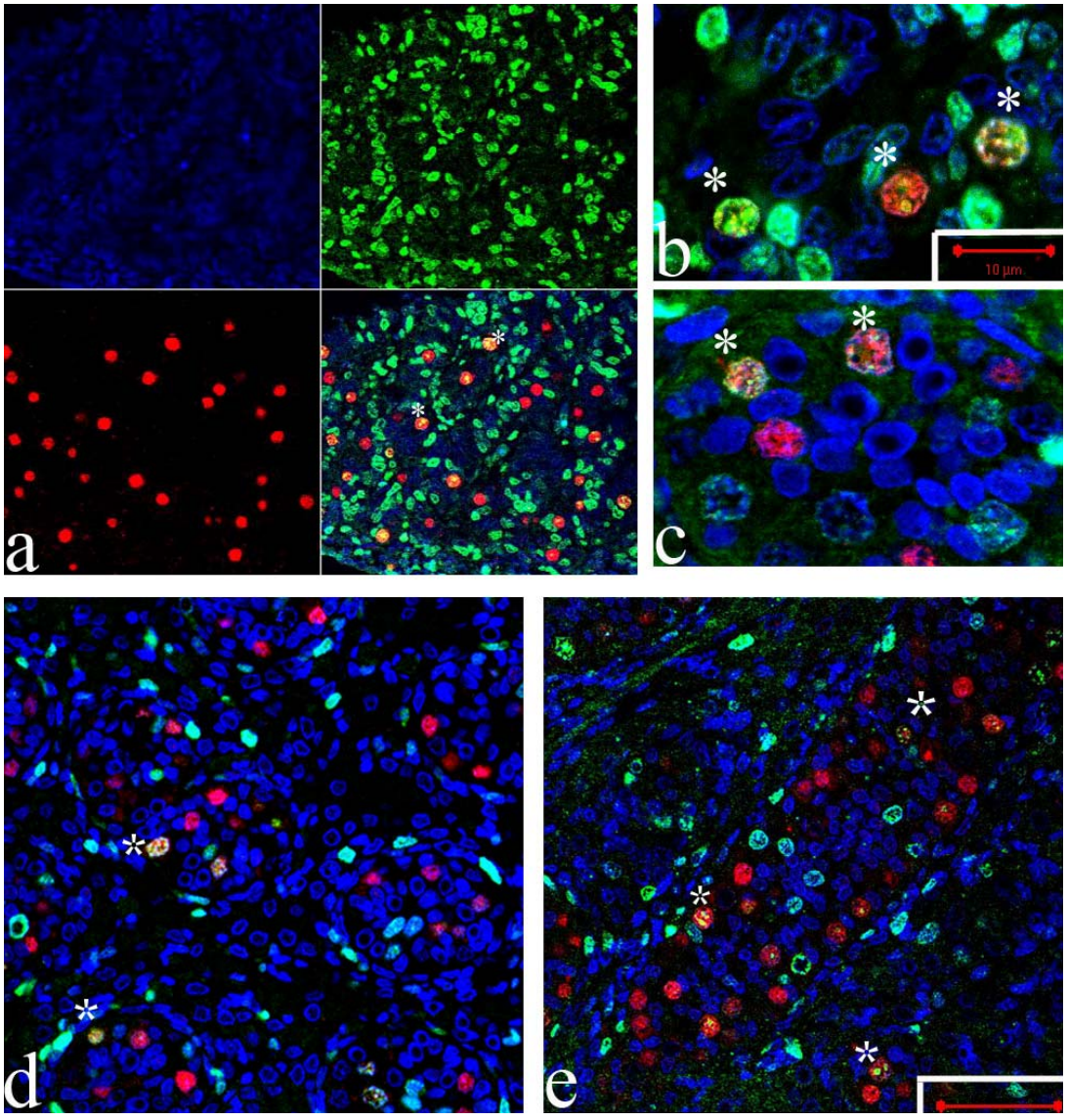


Figure 3-18 Immunofluorescent staining of Ki67 (green) with OCT4 (red) in 1st and 2nd trimester human fetal testes a) 65 days b) high power image of 65 days c) high power image of 16 week testis d) 15 week testis e) 17 week testis. Asterisks identify co-localisation. In b, bar=20 µm and applies to c also, in e bar=50 µm and applies to a and d also.

3.3.16 Immunoflourescent costaining of Ki67 with VASA

Ki67/VASA co-staining showed that some cells positive for VASA were also positive for Ki67 (Figure 3-19, asterisks) and some VASA positive/Ki67 negative cells were also observed.

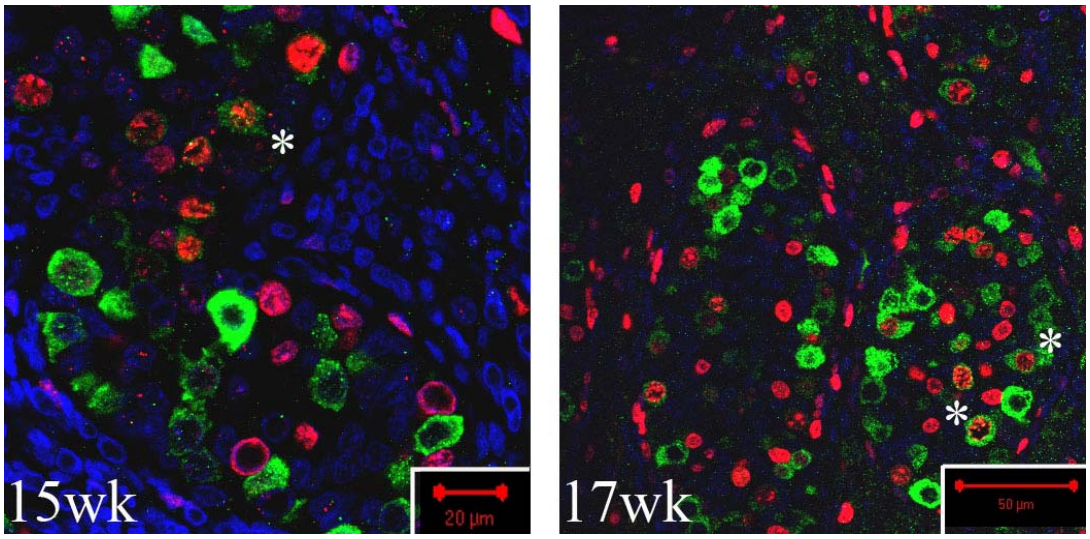


Figure 3-19 Immunofluorescent staining of Ki67 (red) with VASA (green) in 2nd trimester human fetal testis at 15 week and 17 week. Bar=20 µm for 15wk and bar=50 µm for 17 week.

3.4 Discussion

A mixed population of germ cells in the human fetal testis, apparently at differing stages of maturation, have been identified (Fukuda et al., 1975), a situation that appears in contrast to the rodent where germ cells develop in a synchronous fashion. Along with the varying morphological alterations that occur in the germ cells as they mature, there are key changes in protein expression. This study has further characterized the changes in protein expression as germ cells undergo the transition from gonocytes to prespermatogonia.

3.4.1 OCT4 positive gonocytes express AP2γ, DAZL, NANOG and M2A

The transcription factor AP2γ is highly expressed in the OCT4 positive gonocyte population in 1st and 2nd trimester testis. AP2γ appears to be downregulated at around the same time as expression of OCT4 declines, as the VASA positive germ

cells displayed low/negative levels of AP2γ and OCT4 in germ cells of the 2nd trimester testis. Ap2γ is a member of the AP2 family of transcription factors. Studies in the mouse suggest that AP2γ has potential roles in proliferation, apoptosis and differentiation of cells (Jager et al., 2003). In *Ap2γ* knock-out mice trophoectodermal cells fail to proliferate (Auman et al., 2002) and AP2γ is upregulated in a variety of tumours, such as melanomas and breast cancer (Turner et al., 1998). Another protein previously restricted to the gonocyte population, KIT (Gaskell et al., 2004) has been shown to contain putative AP2 binding sites in its promoter (Yamamoto et al., 1993). Our findings agree with a previous study that has localized AP2γ to both the gonocyte and the intermediate cell populations (Pauls et al., 2006). The transcription factor NANOG acts synergistically with OCT4 to maintain pluripotency (Liang et al., 2008; Loh et al., 2006). *Nanog* is expressed in the early germ cells of the mouse (Yamaguchi et al., 2005). In this study, NANOG was detected in all germ cells of the first trimester, but by the second trimester NANOG negative germ cells could be identified. Previous studies have shown NANOG to be highly expressed in CIS cells and also in germ cells of the fetal testes up until 20 weeks gestation, where it appears to be downregulated earlier than OCT4 (Hart et al., 2005; Hoei-Hansen et al., 2005).

In this study, the RNA-binding protein DAZL was detected in germ cells of the 1st and 2nd trimester, where it was located in either the nucleus or cytoplasm, or in some cases both. This is consistent with previous studies that have shown DAZL to be present in the germ cell nuclei of a 17 week human fetal testis (Ruggiu et al., 2000), and others have described DAZL in both the nucleus and cytoplasm of human germ cells (Reijo et al., 2000). The change in subcellular location of DAZL during fetal germ cell maturation is intriguing considering its role as an RNA binding protein. The role of DAZL may be to activate silent mRNAs by recruiting poly A binding proteins, resulting in the expression of a subset of mRNAs during germ cell maturation (Collier et al., 2005). The nuclear location of DAZL in immature human fetal germ cells may be a means of storing DAZL and rendering it inactive. The Drosophila homologue of DAZL, boule, is initially found in the nucleus of

premeiotic germ cells and this translocates to the cytoplasm at the onset of meiosis (Cheng et al., 1998). In the rodent Dazl has only been detected in the cytoplasm of the germ cells, the difference between the rodent and the human may be due to the DAZ gene cluster which is present on the Y chromosome of humans and higher primates (Cooke et al., 1996; Reijo et al., 1995). The functions of DAZ have not been fully established but it has been shown to be capable of partial rescue of the DAZL mutant mouse (Slee et al., 1999).

Previous studies have implicated Dazl in the regulation of Vasa, as *Dazl* knock-out mice display reduced levels of Mvh, while the 3' UTR of *Mvh* contains various Dazl binding sites, and this is conserved among a variety of species (Reynolds et al., 2005). Our study would support this hypothesis given that DAZL is expressed earlier than VASA. DAZL appears to be more of an intermediate marker, remaining expressed in VASA positive cells, although possibly changing from a nuclear location in gonocytes to the cytoplasm in prespermatogonia. The slight increase in DAZL immunoexpression in 2nd trimester testis is in contrast to the situation in the ovary where there was a dramatic increase in DAZL expression in the second trimester at the time when germ cells enter meiosis (Anderson et al., 2007).

The oncofetal marker M2A localizes to the membrane of OCT4 positive gonocytes and is expressed in a distinct population from the VASA positive germ cells. Our findings are consistent with a previous study when M2A was described as an 'early' germ cell marker (Pauls et al., 2006).

3.4.2 OCT4 negative prespermatogonia express VASA and NANOS1

The NANOS1 protein was found to be germ cell specific in the human fetal testis. It was found to be expressed at low levels in fetal testes of the first trimester, and in testes of the second trimester it was found to co-localize with the VASA positive germ cells. In Drosophila, *nanos* is essential for the migration of PGCs (Wang and Lehmann, 1991). However in the mouse, knock-out of *Nanos1* was shown to cause no disruption to germ cell development, while loss of *Nanos2* and *Nanos3* resulted in

germ cell defects, with *Nanos2* -/- mice characterised by a complete depletion of spermatogonia, and *Nanos3* -/- mice exhibiting an earlier loss of germ cells at the PGC stage, in both males and females (Tsuda et al., 2003). This is the first report of NANOS1 expression in the human fetal testis. In our study, NANOS1 was immunolocalised mainly to the nuclear periphery of germ cells, however, the fact that *Nanos1* appears to not be necessary for germ cell development in the mouse raises questions about its role in the human, although a previous group have reported the expression of NANOS1 in the germ cells in adult human testis, where it was also found to be expressed in a perinuclear location (Jaruzelska et al., 2003).

VASA was not detected in human germ cells during the first trimester, this finding is in contrast to reports in the mouse where studies have shown that contact between germ cells and somatic cells within the gonadal ridge is enough to induce Mvh expression (Toyooka et al., 2000). VASA was detected in 2nd trimester testis where it was found to be present in the germ cells that no longer expressed OCT4. NANOS1 appears to be expressed slightly before VASA as it was just detectable in first trimester gonads.

3.4.3 OCT4 positive CIS cells have low/negative levels of VASA

Immunofluorescent co-staining for OCT4 and VASA identified OCT4 positive CIS cells within the tubules of TGCT samples. This is in agreement with previous reports that CIS cells express OCT4 (Looijenga et al., 2003; Rajpert-De Meyts et al., 2004). Other groups have reported the expression of VASA in CIS cells (Honecker et al., 2004; Zeeman et al., 2002). Co-immunostaining for OCT4 together with VASA in CIS cells demonstrated for the first time that CIS cells within the abnormal tubules were negative for OCT4 and conversely that OCT4 positive cells were either VASA immunonegative or expressed the protein at very low levels. There are several possible explanations for the mutually exclusive expression of OCT4 and VASA in CIS cells, one possibility is that CIS cells do not express VASA, and originate from the period in fetal life where germ cells are VASA negative (i.e. the

gonocytes) and that the VASA positive cells could be 'normal' germ cells located within the same tubule. Alternatively, the VASA positive cells within the CIS tubules may also be CIS cells that do not express OCT4, possibly because some CIS cells are themselves capable of some form of limited differentiation. Heterogeneity of CIS cells has previously been reported, where OCT4 expression was described at high levels in a number of cells, and other CIS markers such as C-KIT were expressed in only a subset of these cells (Rajpert-De Meyts et al., 1996). We also have unpublished data showing that C-KIT and MAGE-A4 are not co-expressed.

3.4.4 Germ cells proliferate throughout the 1st and 2nd trimester

In this study, a high number of Ki67 positive germ cells were found in the 1st trimester, although this was not quantified. Proliferation was found to continue throughout the second trimester, with a slight decrease towards the end of the second trimester. This is consistent with work done by Murray et al (2000) who found that germ cells throughout the second trimester expressed the proliferation cell nuclear antigen (PCNA). Previous studies have reported that germ cells proliferate extensively throughout the 1st trimester (Bendsen et al., 2006). In the human, germ cell number has been found to increase exponentially from week 7 to week 19, with a doubling time of 12 days, resulting in an increase in the germ cell to Sertoli cell ratio (O'Shaughnessy et al., 2007). This would explain the increase in protein expression in the second trimester compared with the first for the OCT4, DAZL, AP2γ and NANOS1 Western blots. Honecker et al (2004) reported a decrease in the number of Ki67 positive germ cells throughout the second trimester, with a significant number of immunopositive gonocytes, and then a reduced number of positive cells, until the 3rd trimester when the prespermatogonia become proliferative and remain so until the first weeks of life. This data is consistent with the theory that germ cell proliferation in the human does not cease in the way that occurs in the rodent. For example studies analysing germ cell proliferation in the rat have shown that that germ cell proliferate synchronously at e15.5, where proliferation then stops until the gonocytes form prespermatogonia at postnatal day 4-6 (Ferrara et al., 2006).

3.4.5 Conclusion

In summary, these studies have further expanded the protein profiling of human fetal testicular subpopulations. This chapter has demonstrated for the first time that the OCT4-positive (gonocyte) sub population almost exclusively express the proteins M2A, NANOG, AP2γ and DAZL, while the OCT4-negative prespermatogonia have been shown to express low/negative levels of M2A and AP2γ, while expressing some DAZL and high levels of VASA and NANOS1. In addition, an intermediate population of cells, undergoing the transition form gonocyte to prespermatogonia was also identified. These studies have also shown for the first time that proliferation is not restricted to either the gonocyte or prespermatogonial populations. These observations may be important for the studies on testicular germ cell tumours, as results in this chapter show that within tubules of adult testis containing CIS cells, populations of OCT4 positive/VASA negative and OCT4 negative/VASA positive cells co-exist.

4 Retinoic acid signalling and the control of meiotic entry in the human fetal gonad

4.1 Introduction

During development, germ cells migrate into the gonad by e10.5 in the mouse and by the 5th week of gestation in the human (Wartenberg, 1981). In the ovary, germ cells proliferate and form syncytial nests in close association with the somatic cells, and are surrounded by a network of stromal cells (McNatty et al., 2000). In the mouse, germ cells enter meiosis at e13.5 (McLaren and Southee, 1997), then progress through meiotic prophase I, arresting at the diplotene stage of meiotic prophase by e17.5, forming primordial follicles (Hirshfield, 1992). In the human fetal ovary, germ cells begin to enter meiosis at around 11 weeks gestation (9 weeks post-conception) (Bendsen et al., 2006). Unlike the mouse, germ cells in the human fetal ovary contain oocytes that are present at various stages of meiotic prophase I, with many oogonia continuing to proliferate, while others enter meiosis (Bendsen et al., 2006; Gondos et al., 1986).

In the fetal testis, germ cells become surrounded by the developing Sertoli cells, and once peritubular myoid cells (PTM) migrate into the testis, they surround the Sertoli cells that are organised together with the germ cells to form the testicular cords (Buehr et al., 1993). The interstitial compartment that exists between the cords contains fetal Leydig cells, fibroblasts and blood vessels. In the testis, germ cells do not enter meiosis until puberty, occurring many years after they first arrive in the gonad. In the mouse testis at e13.5, germ cells all arrest in the G1 (G0) stages of the cell cycle as T-prospermatogonia, and then remain in mitotic arrest until one week after birth (McLaren, 1984). In contrast, whilst germ cells in the human fetal testis initially all appear to exist as uniform OCT4+ germ cell population in the 1st trimester, by the 2nd trimester a mixed population of OCT4+ and VASA+ germ cells can be detected within individual cords (Anderson et al., 2007; Gaskell et al., 2004) (Chapter 3). Additionally, unlike the mitotic arrest observed in the mouse testis,

germ cells continue to proliferate throughout the 2nd trimester (Bendsen et al., 2006; Murray et al., 2000) (Chapter 3).

Studies in the mouse ovary have shown that as germ cells enter meiosis, *Oct4* expression is downregulated in a caudal to rostral direction (Pesce et al., 1998; Menke et al., 2003; Bullejos and Koopman, 2004). As *Oct4* expression is downregulated, genes associated with meiosis, such as stimulated by retinoic acid 8 (*Stra8*) and the synaptonemal complex protein 3 (*Sycp3*), are upregulated (Menke et al., 2003; Bullejos and Koopman, 2004). Studies in the human fetal ovary have shown that distinct OCT4 positive/VASA negative populations of cells can be identified, indicating that a similar maturation process occurs in the germ cells of the human fetal ovary as occurs in the fetal testis (Anderson et al., 2007; Stoop et al., 2005)(Chapter 3), and suggests that the downregulation of OCT4 and upregulation of VASA in the human fetal ovary is not linked to meiotic entry. The less mature OCT4 cells were found at the periphery of the ovary, while the more mature VASA positive cells, located within the germ cell nests, were found towards the centre of the gonad (Anderson et al., 2007). Therefore, the maturation of germ cells and meiotic progression in the human fetal ovary may occur differently from the mouse ovary.

The fate of the germ cells and the expression of factors controlling meiotic entry are regulated by the somatic cells of the gonad (refer to Chapter 1, section 1.3.2.5). Some recent progress has been made in understanding mechanisms controlling meiotic entry in the mouse. In the female gonad, expression of *Stra8* is upregulated one day before meiotic entry (Menke et al., 2003). In *Stra8*-deficient ovaries, germ cells develop normally until e13.5, but then fail to initiate meiotic prophase, and deteriorate (Baltus et al., 2006). *Stra8* is a member of a gene family that has been shown to respond to retinoic acid (RA).

RA is the biologically active derivative of retinol (Vitamin A) and has various effects on cells and tissue during development and differentiation (Mendelsohn et al., 1992;

Niederreither et al., 2002; Wendling et al., 1999). The tissue distribution of RA is highly regulated by a group of RA producing and metabolising enzymes. The retinaldehyde dehydrogenase enzymes (Aldh1a 1,2 and 3) are responsible for the oxidation of the RA precursors to produce RA (Maden, 2007; Reijntjes et al., 2005). RA signalling is negatively regulated by three RA degrading enzymes, Cyp26A1, Cyp26B1 and Cyp26C1, which metabolize RA into hydroxylated polar derivatives (Fujii et al., 1997) (Literature Review Section 1.3.5.2.1).

Studies by Koubova et al (2006) found that the normal upregulation of *Stra8* failed to occur when e11.5 ovaries were cultured for two days in the presence an antagonist for all retinoic acid receptors, BMS-204493. In contrast when testes were cultured in the presence of RA, *Stra8* was upregulated, specifically in the germ cells. A further part of the jigsaw was provided by Bowles et al in 2006 who found that the RA metabolising enzyme *Cyp26b1* was initially expressed in the gonads of both sexes, but at e12.5, it became male-specific. This enzyme was demonstrated to be expressed in the testis cords, in the Sertoli cells, with maximal expression in the male at e13.5 (Koubova et al., 2006; Bowles et al., 2006). In ovarian cultures, treatment with RA resulted in a decrease in *Oct4* expression but an increase in *Stra8*; in contrast, incubation with the RA receptor antagonist AGN193109 resulted in a downregulation of meiotic genes, and a sustained expression of *Oct4* (Bowles et al., 2006). Treatment with the cytochrome p450 inhibitor ketoconazole, increased expression of meiotic genes in the testis, and decreased expression of *Oct4* (Bowles et al., 2006). Furthermore, *Cyp26b1* null testis had a 3-fold increase in RA levels and an abnormal number of apoptotic germ cells, with some cells appearing to have entered meiosis. In addition, the enzymes *Aldh1a2* and *Aldh1a3,* responsible for RA biosynthesis, were shown to be expressed within the mesonephroi, but not the gonads, from e10.5-13.5 and high levels of RA were demonstrated to be produced by the mesonephros (Bowles et al., 2006).

A member of the Nanos family of RNA binding proteins also appears to play a role in the suppression of meiosis in the developing testis. In the mouse and human, three

Nanos family members (Nanos 1,2 and 3) have been identified (Tsuda et al., 2003). Nanos2 is expressed specifically in germ cells of the embryonic mouse testis and is absent from the ovaries (Tsuda et al., 2003; Suzuki and Saga, 2008). Nanos2 appears to play an indirect role in the suppression of *Stra8* and therefore contributes to the mechanisms preventing male germ cells from entering meiosis after Cyp26b1 has been downregulated (Suzuki and Saga, 2008). Moreover, Nanos2 may also have a role in committing male germ cells to the spermatogonial lineage, as ectopic expression of Nanos2 in female germ cells induced their differentiation down the male lineage (Suzuki and Saga, 2008).

Despite the convincing evidence that RA may be the signal leading to the initiation of meiotic entry in the developing mouse ovary, there are a few questions which remain about the true function of RA. For example, although other groups have previously reported RA as an important mitogen and survival factor for germ cells, they never described any germ cells displaying signs of meiotic entry (Koshimizu et al., 1995; Morita and Tilly, 1999).

Recently a novel transmembrane protein has been implicated in the commitment of germ cells to the male lineage. Expression of Sdmg1 is specifically upregulated in the Sertoli cells of the embryonic testes at e12.5. Any substance involved in committing the germ cells to the spermatogenic lineage may be secreted by the Sertoli cells at this time, aided by Sdmg1. If secretion from the Sertoli cells is inhibited at e12.5, it results in germ cells undergoing male-to-female sex reversal, with some germ cells appearing to enter meiosis (Best et al., 2008).

4.1.1 Aims of Chapter

The aim of this study was to determine whether a similar system to that reported to control the meiotic progression of mouse germ cells, operates in the human fetal testis and ovary. Expression of key genes documented to be important for entry or inhibition of meiosis were studied in male and female gonads from the 1st and 2nd trimester. We aimed to determine whether enzymes responsible for the production

and degradation of RA were expressed in the ovary, testis and mesonephros, and ultimately whether or not the gonad produces RA.

4.2 Materials and Methods

4.2.1 Collection of human fetal gonads

Human fetal testes and ovaries were obtained following termination of pregnancy during the 1st and 2nd trimester as outlined in section 2.1.

4.2.2 Immunohistochemistry

4.2.2.1 DAB

Immunohistochemistry was performed and is detailed in section 2.2. Information regarding the primary antibodies used is listed in Table 4-1.

Table 4-1 List of antibodies used for immunohistochemistry

Antigen	Species	Source	Antigen Retrieval	Dilution
RARα	Rabbit	SCruz	No	1.50
RARβ	Rabbit	Abcam	Yes	1.100
RXRα	Rabbit	SCruz	Yes	1.100

4.2.3 RNA Extraction

Details on the extraction of RNA from tissue is described in section 2.4.

4.2.4 Preparation of cDNA using random hexamers

cDNA was prepared as outlined in section 2.5.

4.2.5 TaqMan analysis

The Roche Universal Probe Library was used for TaqMan analysis. The procedures for the TaqMan reaction and analysis are detailed in section 2.5. Primers and probes used are listed in Table 4-2. TaqMan analysis was performed in triplicate on 1st

trimester (50-65 days), early 2nd trimester (14-15wks) and late second trimester (18-19wks) ovaries and testes (N=4 for each age group).

Table 4-2 Summary of primers and probes used for the TaqMan reaction

Gene name	5' sequence	3'sequence	Probe
STRA8	ggccttagctgtgcaaacac	ctcttcatcaacgggaaagg	4
NANOS1	tgaaagaaaaggtgcatttcaa	cctggctaagaaacattgtgc	51
NANOS2	gtcttcgcaggctcacct	ggcattgaaaggtgtcagc	58
NANOS3	gcagggttacttgtctctgtga	acaggtcaaaggtccccata	44
RARα	cagcaccagcttccagttagt	agaactgctgctctgggtct	83
RARβ	cacctgtcatcggaggactt	ggtgctctgtgtttcaattgtt	16
RARγ	cgctccatccaagagactg	gagggaactgggccgtag	70
RXRα	aagcggatcccacacttct	gaaggaggcgatgagcag	18
RXRβ	ggcggagaacaacaaacc	gtctgggcttcgggagtc	36
RXRγ	tgtcatgggcatgaagagg	cctcactctcagctct	82
ALDH1A2	ccacagtgttttccaacgtc	tcctgaacagggccaaag	63
ALDH1A3	ctggatgccctgagtcgt	ccctgtatccatcgtctcca	22
CYP26A1	gcagccacatctctgatcact	tgttgtcttgattgctcttgc	45
CYP26B1	acatccaccgcaacaagc	ggatcttgggcaggtaactct	41
CYP26C1	gccctcgacctaatcattca	gagctccacagccgactc	17
SDMG1	cagctaggcttccctggag	aggatccctgagacattactcatc	4

4.2.6 Statistical analysis

Statistical analysis was performed using a Student's t-test and a one-way analysis of variance (ANOVA), followed by a Bonferonni multiple comparison test.

4.2.7 Retinoic acid reporter cells

4.2.7.1 Culture of Retinoic acid reporter cells

The RA reporter F9 teratocarcinoma transformed cell line was kindly donated by Dr Michael Wagner (SUNY Downstate Medical Center, Brooklyn, N.Y., USA). These cells had been transfected with a plasmid reporter construct containing a single copy of the RA response element (RARE) upstream of the Ecoli LacZ gene (Wagner et al., 1992). Upon stimulation with RA, the RARE becomes activated leading to LacZ gene expression. Cells were cultured on 0.1% gelatin coated plates and reached confluence every 2-3 days. The recipe for the medium used for the RA cells is detailed in section 2.7

4.2.7.2 Incubation of tissue with retinoic acid reporter cells

Reporter cells were plated onto a 24 or 48 well plate at 70% confluency. Gonadal tissues were isolated from human fetuses during the 1st and 2nd trimester or mouse embryos (C57BL/6) at e11.5 (N=4) and e13.5 (N=5). For 1st trimester human fetal gonads and embryonic mouse testes, mesonephroi (N=2) were separated from the gonads. Tissues were incubated with the reporter cells for 24 or 48 hours. As a positive control, Ion-Exchange bead (Biorad) were loaded with 1x10^{-10}M of RA (all trans, Sigma) for 1 hour, and then washed 3x10 minutes in PBS, before being incubated with the cells in a separate well for the same length of time as the tissues.

4.2.7.3 βGalactosidase staining

βGalactosidase (βGal) staining was performed to visualise the cells that expressed LacZ. The βGal enzyme is encoded by the LacZ gene. βGal cleaves the X-gal substrate yielding galactosidase and 5-bromo-4-chloro-3-hydroxyindole. The latter is further oxidised to an insoluble blue product, 5,5'-dibromo-4,4' dichloro-indigo. The βGal staining kit was obtained from Invitrogen and staining was performed in accordance with manufactures instructions (Invitrogen Cat # K1465-01). Briefly, cells were washed in PBS, and fixed in the fixative solution provided for 10 minutes. Cells were then washed in PBS again and incubated with the X-gal containing

solution provided. Cells were incubated overnight at 37°C. βGal positive cells displayed blue staining.

4.2.7.4 Fluorescein di-β-D-galactopyranoside (FDG)

Fluorescein di-β-D-galactopyranoside (FDG) is a sensitive alternative for βGal. FDG is hydrolyzed by β-gal, first to fluorescein monogalactoside and then to the highly fluorescent fluorescein. Cells were washed in PBS and then incubated in 50% PBS, containing 1 mM FDG (Invitrogen, Cat # F1179) for 1 minute at 37°C. This solution was removed, followed by incubation with ice-cold PBS for 1 hour on ice before being visualized using an Axiovert 200 (Carl Zeiss).

4.3 Results

4.3.1 Differential expression of *STRA8* in male and female gonads

To investigate the expression of *STRA8* in the human fetal testes and ovaries, TaqMan quantitative PCR analysis was performed for RNA extracted from testes and ovaries of the 1st trimester (50-65 days), early 2nd trimester (14-15 weeks), and late 2nd trimester (18-19 weeks). *STRA8* mRNA expression was low/negative in the 1st and 2nd trimester testes, but was expressed at high levels in the ovaries all the gestational ages examined. *STRA8* expression was significantly higher in 2nd trimester ovaries compared with testes in both the early and late stages ($P<0.05$) (Figure 4-1). *STRA8* expression in the early second trimester ovaries was significantly higher than in the first trimester ovaries ($P<0.0001$). The expression of *STRA8* was slightly lower in ovaries of the late second trimester compared with the early second trimester ($P<0.05$) (Figure 4-1).

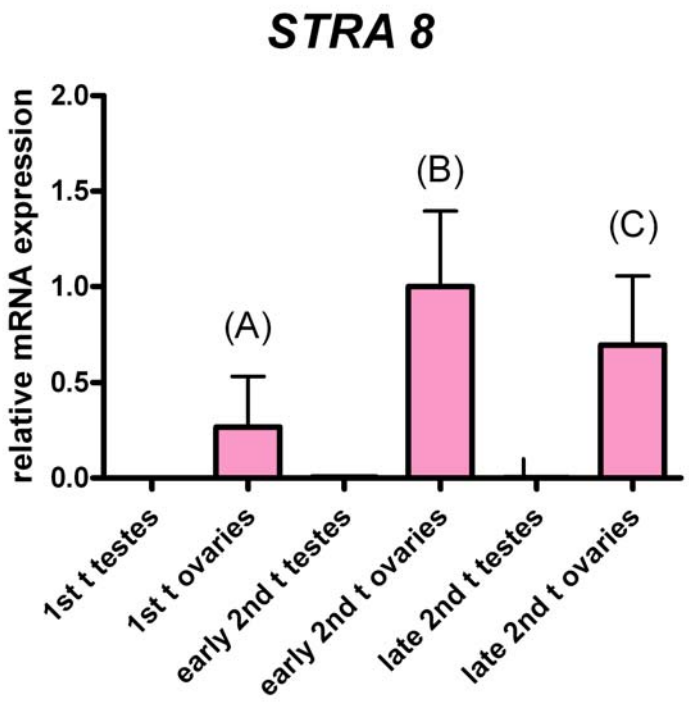


Figure 4-1 STRA8 mRNA expression in male and female gonads during the 1st and 2nd trimester relative to early second trimester ovary. A=Expression in 1st trimester ovaries was significantly lower than early 2nd trimester ovaries (P<0.05). B=mRNA expression in early 2nd trimester ovaries were significantly higher than early 2nd trimester testes (P<0.0001). C=mRNA expression in late 2nd trimester ovaries was significantly higher than late 2nd trimester testes (P<0.0002). Taqman was performed in triplicate for four independent fetuses in each group. Mean ± sem

4.3.2 Analysis of NANOS gene expression in male and female gonads

To determine whether the NANOS family of genes were expressed in the fetal gonads, and if so, whether they were expressed in an age-specific or sex-specific manner, NANOS 1, 2 and 3 mRNA concentrations were analysed. Analysis of *NANOS1* mRNA expression in 1st and 2nd trimester ovaries and testes revealed that expression was relatively constant throughout the 1st and 2nd trimester, for both the testes and ovaries (Figure 4-2a).

NANOS2 mRNA expression was barely detectable in 1st and 2nd trimester ovaries, and whilst mRNA concentrations were low in 1st trimester testes, concentrations

increased in the early 2nd trimester, and by the late 2nd trimester, levels were significantly higher than the ovaries ($P<0.001$) (Figure 4-2b). *NANOS3* mRNA was detectable at all time points examined in both male and female gonads. *NANOS3* mRNA expression was always higher in the ovaries than the testes for each age group examined (Figure 4-2c). There was therefore a large increase in *NANOS2* expression in the testis but not the ovary, while there was no significant difference in NANOS1 or NANOS3 expression in either sex.

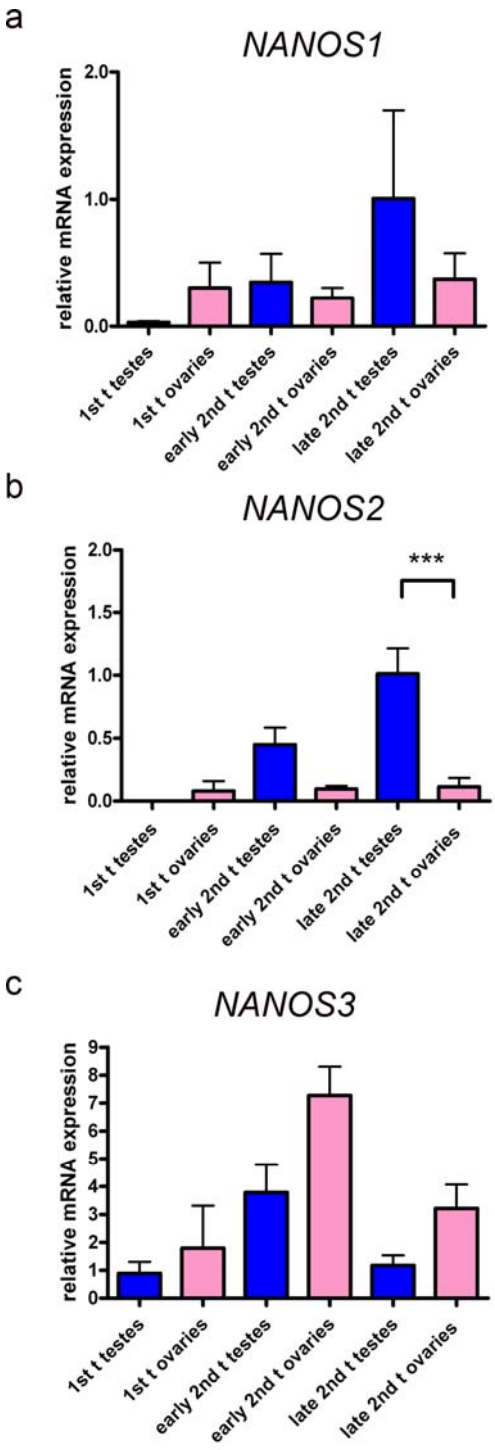


Figure 4-2 mRNA expression of a) NANOS1 b) NANOS2 c) NANOS3 in human fetal gonads during the 1st and second trimester. N=4, relative to late 2nd trimester testis value. Taqman was performed in triplicate for four independent fetuses in each group. Mean ± sem

4.3.3 Expression of RARs and RXR in the human fetal ovaries and testes

To determine whether the RA receptors were present in the male and female gonad, and to analyse any changes in gene expression which occurs with development, TaqMan analysis for the RARs and RXRs was performed. mRNA expression of all of the RARs and RXRs could be detected in the 1st and 2nd trimester ovaries and testes. *RARα* was expressed at relatively constant levels in both the male and female gonads throughout the developmental stages examined (Figure 4-3a). *RARβ* mRNA levels were highest in the 1st trimester for both the ovaries and testes, but mRNA levels at this time were indistinguishable between the sexes. In the 2nd trimester, expression declined compared with the first trimester; this was more marked in the ovaries compared with the testes (Figure 4-3b), but this also was non-significant. *RARγ* mRNA expression peaked during the early 2nd trimester in the ovaries, while expression in the testes tended to be higher in the 1st trimester (Figure 4-3c).

RXRα mRNA was expressed in both the ovaries and testes at similar levels during the 1st trimester, expression rose slightly in the ovaries during the early 2nd trimester in the ovaries, and then decreased towards the end of the 2nd trimester (Figure 4-3d). Messenger RNA levels for *RXRβ* were similar in both the testes and ovaries during the 1st trimester. In the 2nd trimester testes, *RXRβ* mRNA remained at similar levels. In the 2nd trimester ovary, *RXRβ* mRNA levels increased (Figure 4-3e). *RXRγ* mRNA levels were maintained at similar levels in both the male and female at all ages examined (Figure 4-3f).

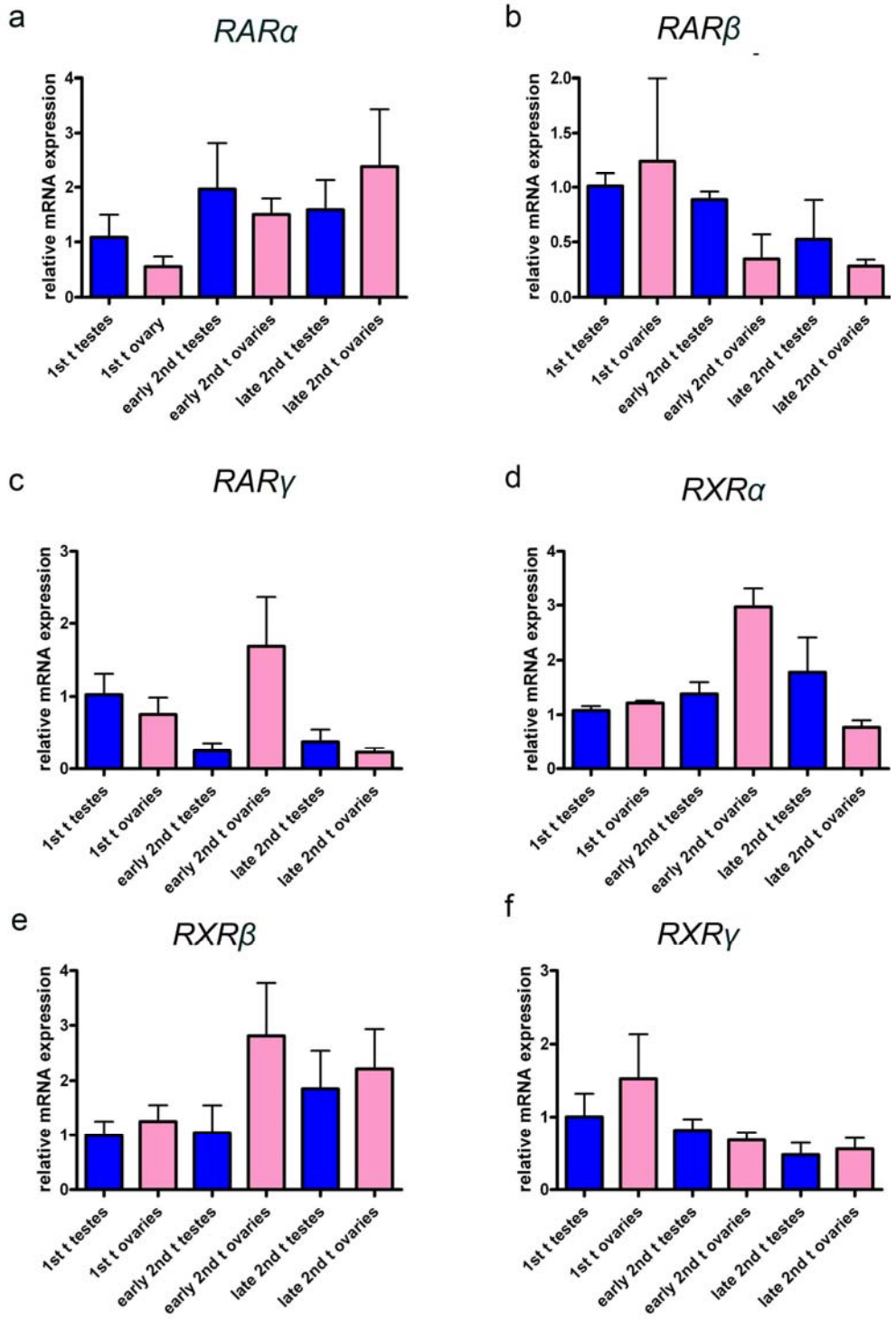


Figure 4-3. mRNA expression of RARs and RXRs in human fetal testis and ovaries a) RARα b) RARβ c) RARγ d) RXRα e) RXRβ f) RXRγ. N=4, all relative to 1st trimester testis. Taqman was performed in triplicate for four independent fetuses in each group. Mean ± sem

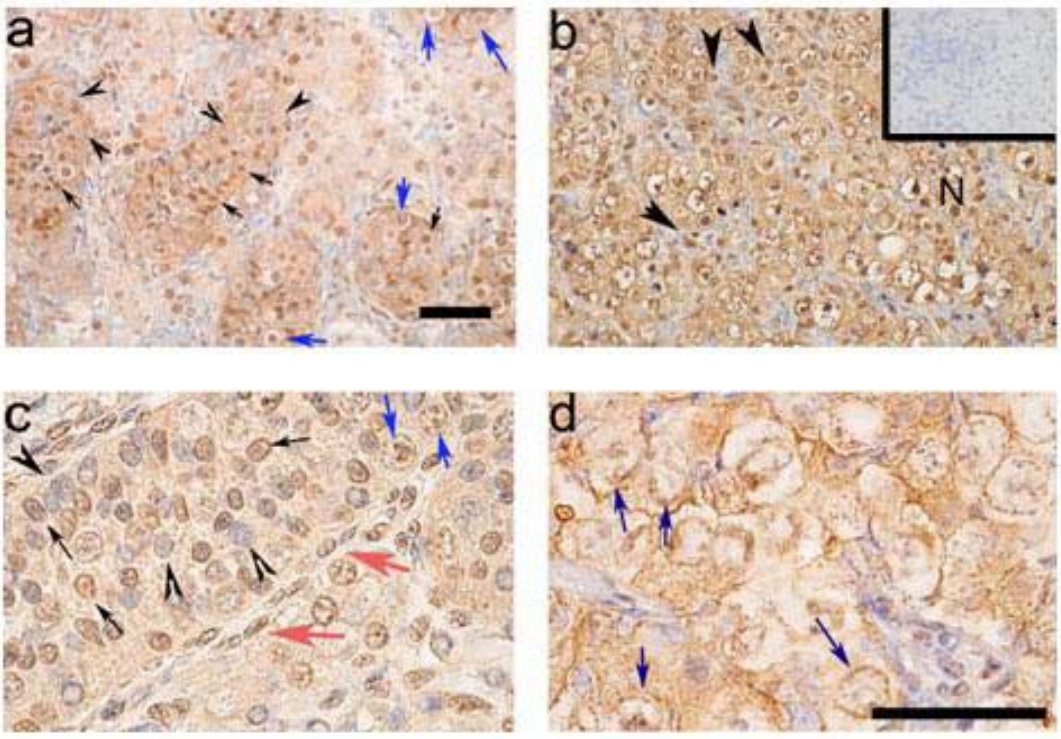


Figure 4-4 RARα immunoexpression in a) 16 week testis b) 14 week ovary c) 18 week testis d) 18 week ovary Bar = 50 µm. In a) and c) black arrows point to immunopositive Sertoli cells. Arrowheads point to immunonegative Sertoli cells, red arrows identify immunopositive PTM cells. In b) arrowheads point to immunopositive somatic cells, N indicates nests of germ cells and in a, c and d) blue arrows show immunopositive germ cells. Inset shows negative control.

To determine whether any protein for the RARs and RXRs were present, and if so, what their distribution within the cells of the ovary and testis was, immunohistochemical analysis was performed using antibodies directed against, RARα, RARβ and RXRα. In the 2nd trimester human fetal testis RARα protein was immunolocalised to the nuclei of some but not all of the Sertoli cells (Figure 4-4 a and c, arrows), with a subpopulation being immunonegative (Figure 4-4 a and c, arrowheads). Some PTM cells were also immunopositive for RARα (Figure 4-4 a and c, red arrows), as were cells within the interstitium. Immunopositive germ cells could also be detected in the testis (Figure 4-4 a and c, blue arrows) In the 2nd

trimester ovaries RARα staining was present within the nests of germ cells, where it was immunolocalised to the nucleus and the cytoplasm Figure 4-4 b and d, blue arrows). The nuclei of the somatic cells in association with the germ cells also stained positive for RARα (Figure 4-4b and d, arrowheads)

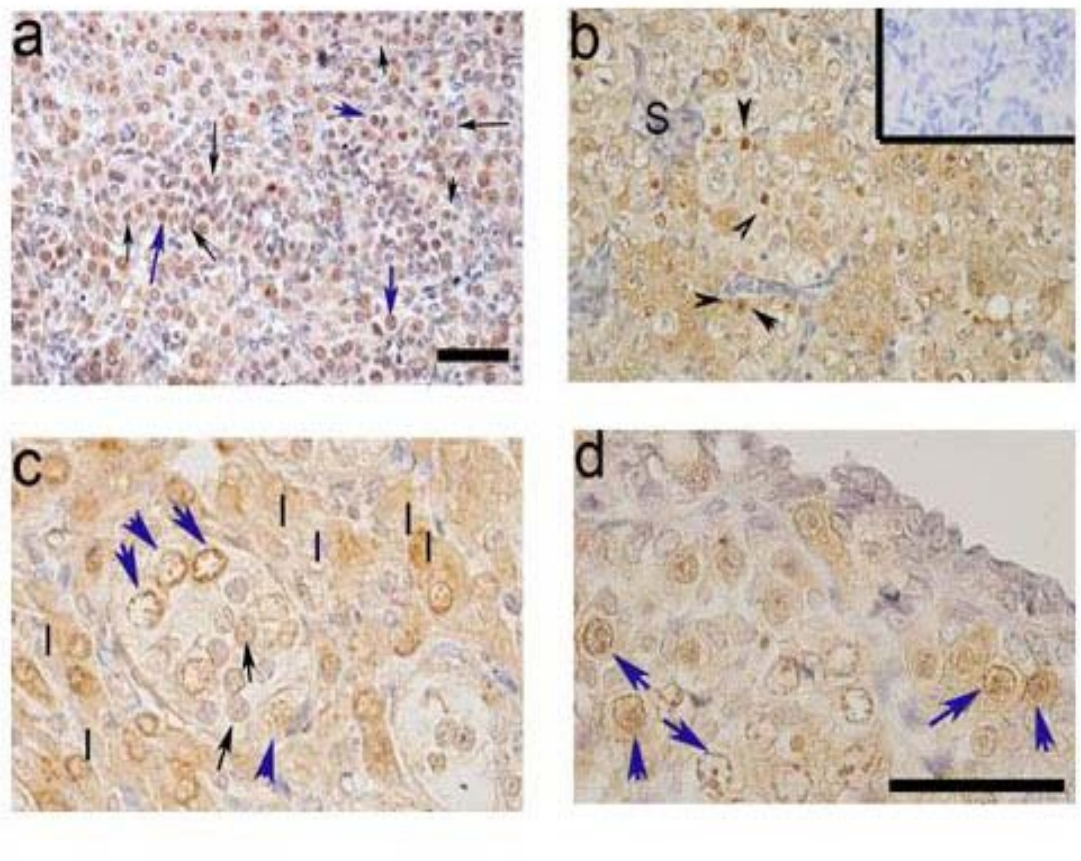


Figure 4-5 Immunostaining for RARβ in 2nd trimester human fetal ovaries and testes. a) 14 week testis b) 18 week ovary c) 15 week testis d) 18 week ovary. Bar = 50 µm. For a) and c) arrows point to immunopositive Sertoli cells, blue arrows identify immunopositive germ cells and I indicates the interstitium where immunpositive Leydig cells can be identified. In b) and d) arrowheads point to immunopositive ovarian somatic cells, blue arrows indicate immunopositive germ cells and S indicates stromal cells. Inset shows negative control.

Immunostaining for RARβ in 2nd trimester human fetal testis showed that a large number of cells within the interstitium were immunopositive for RARβ (Figure 4-4a

and c). The majority of Sertoli cells were also immunopositive (Figure 4-4a and c, arrows) and a number of germ cells also displayed positive staining (Figure 4-4a and c, blue arrows). PTM cells appeared to be largely immunonegative. In the 2nd trimester human fetal ovary, a number of germ cells exhibiting positive staining could also be identified (Figure 4-4 b and d, blue arrows). Immunostaining was found in both the nucleus and the cytoplasm of these cells.

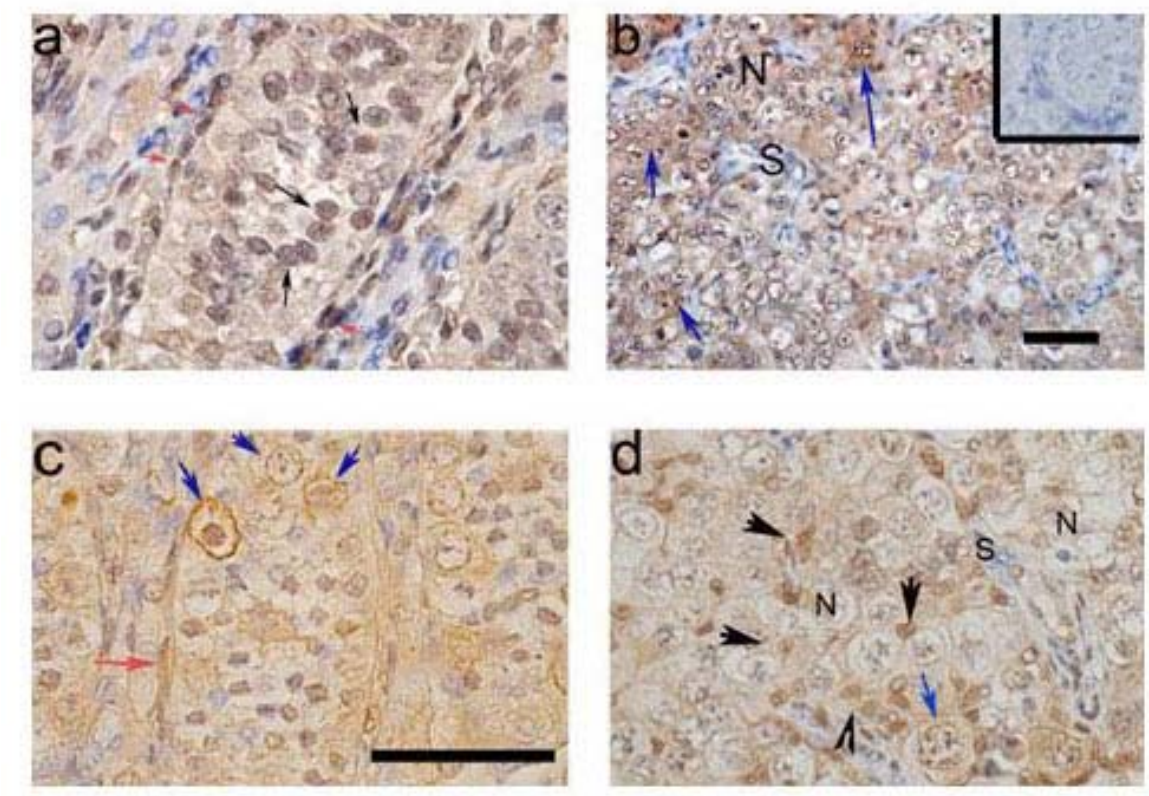


Figure 4-6. RXRα immunostaining in 2nd trimester ovaries and testes. a) 16 week testis b) 19 week ovary c)15 week testis d) 19 week ovary . Bar = 50 µm. For a) and c) black arrows point to immunopositive Sertoli cells, and red arrows point to immunopositive PTM cells, blue arrows denote immunopositive germ cells. For b) and d) N indicates nests of syncytial germ cells, S indicates stromal cells and blue arrows show immunopositive germ cells. Inset shows negative control.

Immunhistochemical staining of RXRα showed that in the 2nd trimester testis, the majority of Sertoli cells were immunopositive (Figure 4-6a, arrows), as were PTM cells. RXRα staining was also identifiable in some of the germ cells (Figure 4-6 a and c) where it was found mainly in the cytoplasm. In the ovary, RXRα was detected within the germ cell nests, where it appeared to be present within the cytoplasm and nuclei of some of the germ cells, and the interstitial cells were immunonegative (Figure 4-6 b and d).

4.3.4 Expression of RA producing enzymes in the human fetal gonad and mesonephros

To determine whether all or some of the enzymes involved in the production of RA were present within the gonad or mesonephros, TaqMan analysis was performed to examine the levels of transcripts for the *ALDH1A2* and *ALDH1A3* genes. Total concentrations of *ALDH1A2* mRNA were lower in the mesonephros than in the gonads. In 1st trimester testis, concentrations of *ALDH1A2* mRNA were higher than in the testis recovered during the 2nd trimester. In the ovary, mRNA concentrations were maintained at a similar level at all stages of gestation examined. Expression in the 2nd trimester ovaries was higher than in the 2nd trimester testes (Figure 4-7a). *ALDH1A3* mRNA levels were also lower in the mesonephros than the male and female gonads. In the ovaries and testes during the 1st trimester and early 2nd trimester *ALDH1A3* mRNA expression was similar (Figure 4-7b).

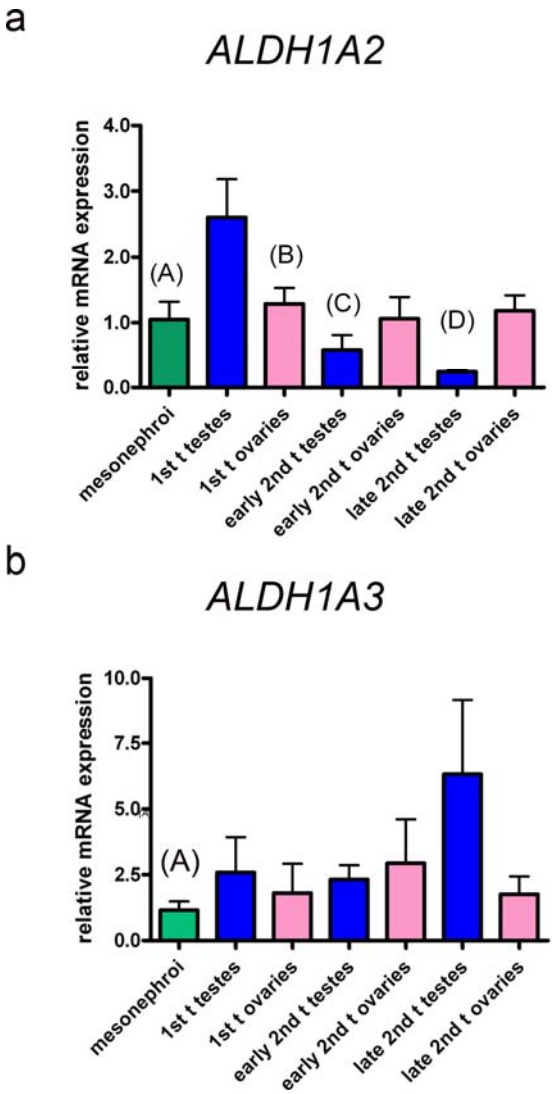


Figure 4-7 mRNA expression of ALDH1A2 and ALDH1A3 in human fetal mesonephroi and gonads (N=4). Both relative to mesonephroi. a) For ALDH1A2, A= mRNA expression in the mesonephroi was significantly lower than 1st trimester testes (P<0.01). B= mRNA levels in 1st trimester ovaries were significantly lower than 1st trimester testes (P<0.05). C= early 2nd trimester testes mRNA expression was significantly lower than 1st trimester testes. D= late 2nd trimester testes was significantly lower than 1st trimester testes (P<0.05). For b) A=ALDH1A3 mRNA levels in the mesonephroi was significantly lower than the late 2nd trimester testes (P<0.05). Taqman was performed in triplicate for four independent fetuses in each group. Mean ± sem

4.3.5 Analysis of human fetal mesonephroi and gonads for the production of retinoic acid using a retinoic acid reporter cell line

Having detected the mRNA expression for the genes responsible for the production of RA, we next wanted to determine whether RA was actually being produced. In order to establish whether human fetal mesonephroi or gonads produced RA, tissues were cultured on a layer of RA responsive cells and a βGal assay was performed. Mouse mesonephroi and RA containing ion-exchange beads were used as a positive control.

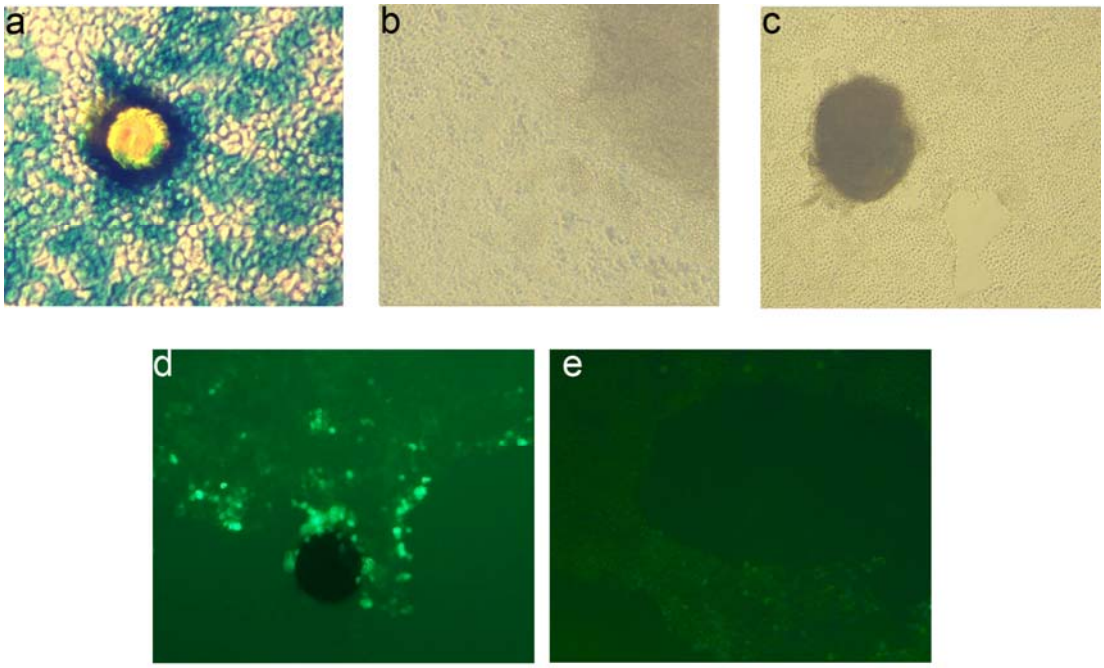


Figure 4-8 RA reporter cell assays. a) βGal assay showing cells incubated with RA loaded ion-exchange bead b) βGal assay following incubation with human mesonephroi at 8 weeks gestation c) βGal assay for cells incubated with mouse mesonephroi at e13.5 d) FDG assay for ion exchange beads e) FDG assay for mouse mesonephroi at e13.5 following 24 hours of culture.

Analysis of RA reporter cells following culture of tissues after 24 and 48 hours showed that no βGal positive cells could be detected in cultures containing mouse mesonephroi, or human mesonephroi, while βGal positive cells could be detected in cultures with beads containing $1x10^{-10}$M RA (Figure 4-8a-c). To determine if a stronger response could be obtained from a more sensitive method, an FDG assay

was performed. Cells cultured in the presence of RA containing ion exchange beads were positive for fluorescein, while cultures containing mouse mesonephroi were negative (Figure 4-8d and e), thus showing a similar response as with the βGal assay. In both assays only some of the RAR-LacZ cells appear to be responding when cultured in the presence of the RA containing ion exchange beads, this is unlikely to be the result of diffusion gradients and suggests that a heterogenous population of reporter cells are present with some cells having lost expression of the transgene.

4.3.6 mRNA expression of Cytochrome 26 in the human fetal gonads

To obtain an understanding of the expression pattern of the Cyp26 enzymes in the human fetal gonads, TaqMan analysis was performed for mRNAs encoded by the *CYP26A1, CYP26B1* and *CYP26C1* genes. Both *CYP26A1* and *CYP26C1* were undetectable in ovaries and testes examined, at all age groups. But in the positive control, the human endometrial adenocarcinoma Ishikawa cell line, low levels of transcript could be detected (Figure 4-9 a and c).

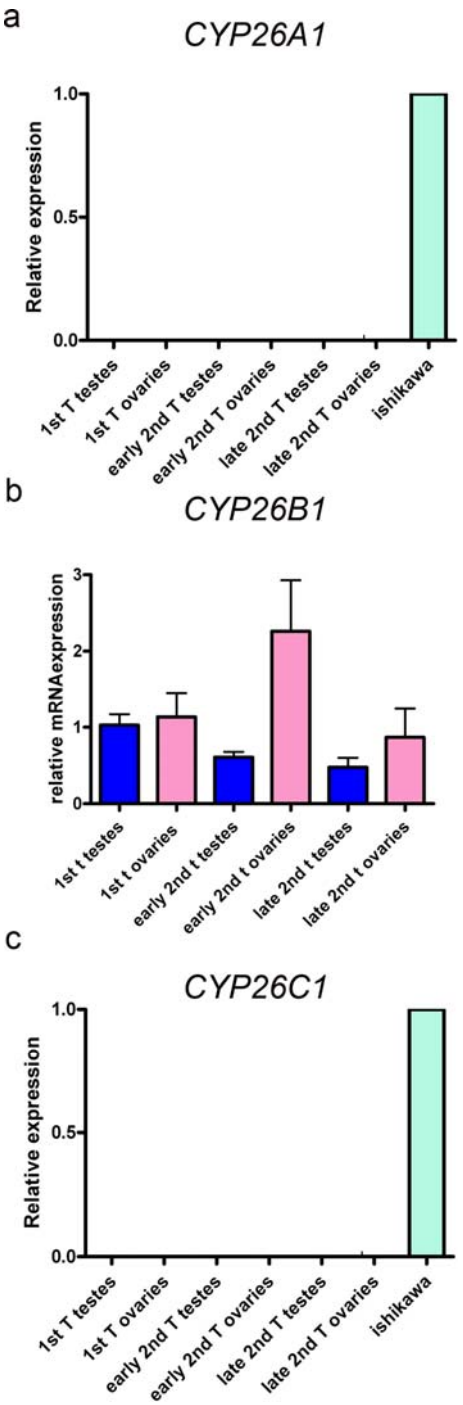


Figure 4-9 mRNA expression of a) CYP26A1 in human fetal gonads and Ishikawa cells b) CYP26B1 expression in human fetal gonads, relative to 1st trimester testes c) CYP26C1 in human fetal gonads and Ishikawa cells. Taqman was performed in triplicate for four independent fetuses in each group. Mean ± sem

In both the 1st and 2nd trimester ovaries and testes, *CYP26B1* mRNA levels were found to be similar. mRNA levels then subsequently declined in the ovary toward the end of the 2nd trimester. mRNA levels in the testes were maintained at a similar levels throughout the 2nd trimester (Figure 4-9) .

4.3.7 Analysis of *SDMG1* mRNA expression in human fetal ovaries and testes

Analysis of *SDMG1* mRNA expression in human fetal gonads was performed. The overall expression of *SDMG1* was higher in the testes than the ovaries, and in the testes there was a progressive increase in expression with gestational age, while in the ovary, the levels of expression did not change throughout the age-groups analysed (Figure 4-10). In the late 2nd trimester testes mRNA levels were significantly higher than the early 2nd trimester ($P<0.05$), and mRNA levels in the late 2nd trimester testes were significantly higher compared with the late 2nd trimester ovaries ($P<0.001$) (Figure 4-10).

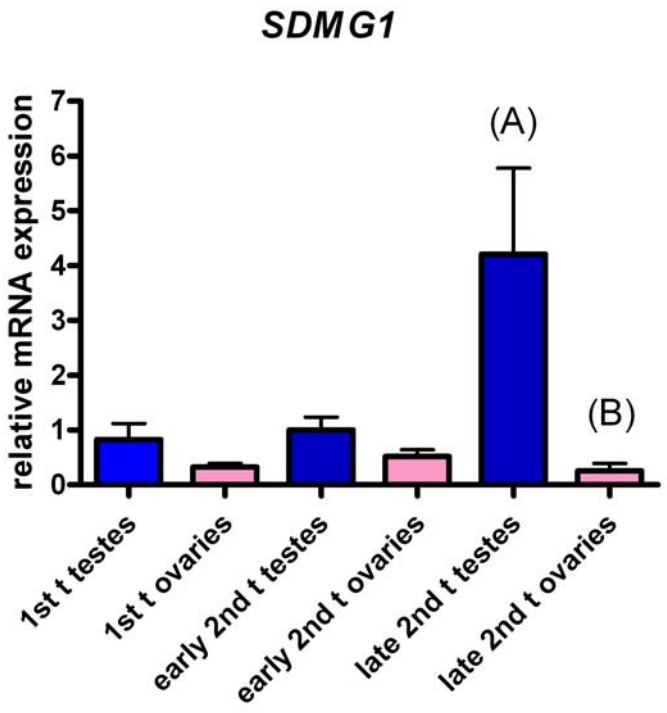


Figure 4-10 SDMG1 mRNA expression in human fetal gonads. A=mRNA levels in late 2nd trimester testes is significantly higher than early 2nd trimester testes (P<0.05). B=mRNA levels in late 2nd trimester ovaries is significantly lower than late 2nd trimester testes (P<0.001) (N=3, relative to early 2nd trimester testis). Taqman was performed in triplicate for four independent fetuses in each group. Mean ± sem

4.4 Discussion

4.4.1 Conserved expression patterns of NANOS2 and STRA8

Expression of *STRA8* mRNA was significantly higher in ovaries compared with testes. In the female *STRA8* mRNA was highest during the early 2nd trimester at a time when germ cells are actively entering meiosis. *STRA8* mRNA was almost undetectable in the testes at all gestational ages examined. We believe that this is the first study that has examined the expression of *STRA8* in the human fetal gonad. *Stra8* was initially identified as a retinoic acid responsive gene, expressed specifically in premeiotic germ cells of the mouse (Miyamoto et al., 2002; Oulad-

Abdelghani et al., 1996; Zhou et al., 2007). This gene has since been shown to be required for germ cells to enter meiosis in both male and female mice (Baltus et al., 2006).

The Nanos gene family of RNA binding proteins were initially identified for their role in germ cell development in Drosophila, where nanos is necessary for the migration and development of germ cells in Drosophila embryos (Forbes and Lehmann, 1998; Kobayashi et al., 1996). In the mouse the *Nanos* gene has been cloned, as have 2 homologues (*Nanos 2 and 3*) (Tsuda et al., 2003). In this study the expression of mRNAs encoded by the Nanos genes was analysed. *NANOS1* was found to be expressed in both the male and female gonads. The total concentration of mRNAs was similar for both sexes until the late second trimester, where the expression of *NANOS1* increased in the male. This supports the results found in Chapter 3 where NANOS1 was found to be specifically localised to the VASA-positive prespermatogonia in the human fetal testis and the large increase can be attributed also to the increase in germ cell numbers with increasing gestation. In the human NANOS1 may have a role in germ cell development, as it has been shown to bind to human PUMILIO2, which is also expressed in spermatogonia (Jaruzelska et al., 2003). In the current study analysis, *NANOS3* mRNA was also detected in male and female germ cells, where expression of NANOS3 was found to peak during the early 2nd trimester in both sexes. In the mouse, Nanos3 is specifically expressed in the germ cells after e7.25 (Yabuta et al., 2006), while *Nanos3*-/- mice display a complete loss of germ cells in both males and female (Tsuda et al., 2003) and the protein appears to be necessary for the maintenance of the germ cell lineage through the suppression of apoptosis (Suzuki et al., 2008). After birth, Nanos3 expression is restricted to the testis, where it has recently been shown to be important for maintaining the undifferentiated state of the spermatogonia via regulation of the cell cycle (Lolicato et al., 2008). Our results would therefore suggest that as in the mouse NANOS3 may play a role in the maintenance of the germ cell population during fetal life in the human male gonad.

In this study, *NANOS2* was sexually dimorphic with significantly higher mRNA levels in the fetal testes than in the female gonads during the 1st and 2nd trimesters. Nanos2 is expressed specifically in germ cells of the mouse testis (Tsuda et al., 2003; Suzuki and Saga, 2008), where it appears to contribute to the suppression of *Stra8* and therefore ensures male germ cells do not commit to meiosis after *Cyp26b1* has been downregulated (Suzuki and Saga, 2008). The male-specific upregulation of *NANOS2* in the 2nd trimester testis suggests that the roles of *NANOS2* may be conserved in the human and may be important for initiating and or maintaining development of testicular germ cells.

Our studies so far have demonstrated the expression of *STRA8* and the *NANOS* genes in the human fetal gonads, but have failed to localise the expression of any of these genes to particular cell types within the gonads. Therefore in situ analysis or immunohistochemical analysis must be performed, firstly to demonstrate germ cell specificity, and secondly to determine which particular germ cells these genes are expressed in, in order to ascertain whether expression patterns of these genes are trully conserved between the mouse and human. It is also important to localise the expression of *STRA8* and the *NANOS* gene family to the cell types of the ovaries and testes, as germ cell number changes dramatically from the first trimester to the second trimester, and analysis of gene expression on RNA extacted from whole gonads is not sufficient to truly understand the changes that occur in gene expression with development.

4.4.2 Both the testis and ovary express the RARs and RXRs during the first and second trimester

In this study, measurements of the mRNAs for the RARs and RXRs in extracts of whole ovaries and testes revealed that all six receptors were present throughout the development stages examined. The RARs are activated by all-trans RA and 9-cis RA, while the RXRs are only activated by 9-cis RA (Allenby et al., 1993). In addition to forming heterodimers with the RARs, the RXRs are also capable of heterodimerizing to other nuclear receptors such as the peroxisome proliferator

activated receptors (PPARs) (Huang, 2008; Kliewer et al., 1992). Immunohistochemistry suggested that RARα was differentially expressed in the nuclei of the Sertoli cells and the germ cells of the testis in the 2nd trimester and was also present within the cells of the interstitium. In the ovary, RARα was detected within the germ cells nests, where it was localised to both the nuclear and cytoplasmic compartments of the germ cells and also to the nuclei of the somatic cells. RARβ protein was also detected in the cells of the testes and ovaries. In the testes, the majority of Sertoli cells were found to express RARβ within the nuclear compartment and RARβ was also localised to both interstitial cells and germ cells. In the developing ovary, RARβ was localised to the germ cells. In the 2nd trimester testes RXRα was expressed in the Sertoli cells, PTM cells, germ cells and interstitial cells, while in the ovary RXRα was localised to the somatic cells in close association with the germ cells. The expression of a number of these receptors in the human fetal gonad makes the formation of different heterodimers a possibility within different cell types of the ovary and testis. For example, in this study, germ cells of the human fetal ovary have been shown to display nuclear expression of RARα and RARβ, making it possible that they are acting as heterodimers.

Expression of RARs and RXRs has been well documented in postnatal rodent testes (Dufour and Kim, 1999; Vernet et al., 2006). The only study into the expression of the RA receptors on the fetal testis has been performed in the rat. Rarα and Rarβ were both reported to be expressed in the interstitial tissue from e14.5 and in the gonocytes from e20.5. Rarγ was undetectable until after birth. Rxrα was localised to the gonocytes from e13.5 and the Leydig cells at e16.5. Rxrβ was detected in the Leydig cells throughout development and Rxrγ was expressed in a variety of cells from e20.5 (Boulogne et al., 1999). A previous study has immunolocalised the RA receptors to the germ cells in the fetal mouse ovary (Morita and Tilly, 1999). Interestingly, the majority of the RARs were detected within the cytoplasm of the germ cells and upon *in vitro* treatment with RA, the RARs redistributed to the nucleus. In this study, the nuclear localisation of some of these receptors in certain cell types suggests that the RA receptors may possibly be signalling within cells of

the gonad. The fact that some of the receptors were found to be nuclear in germ cells of the human fetal testis may suggest that retinoids may be signalling within the germ cells. This is in contrast to the findings of a study into retinoic acid receptor expression in the fetal and neonatal rat testis by Boulogne et al. in 1999, where the receptors were found predominately in the cytoplasm of the germ cells.

The expression of these receptors within the human fetal ovary and testis suggests that RA may have the potential to signal within cell types of the testis, and alter gene expression. The expression of the RA receptors in germ cells means that germ cells may be direct targets for retinoid action.

The potential effects of RA may not only be confined to the germ cells. One previous study used explant cultures to show that RA stimulation results in an increase in steroidogenic function in human fetal 1st trimester testis, while having a negative effect on the number of germ cells (Lambrot et al., 2006a). This study has localised RARα, RARβ and RXRα to the interstitium of the testis, and suggests that the Leydig cells, could be direct targets for RA signalling.

4.4.3 Assessment of the ability for local RA production

The enzymes ALDH1A1, 2 and 3 are responsible for the production of RA through the oxidation of retinaldehyde (Reijntjes et al., 2005; Maden, 2007). This study has shown for the first time that both *ALDH1A2* and *ALDH1A3* mRNAs were detected in the human ovaries, testes and mesonephroi.

In the mouse, *Aldh1a2* and *Aldh1a3* mRNAs have been detected in the mesonephroi of both males and females at e13.5 at a time when meiotic entry occurs in the female germ cells (Bowles et al., 2006). In our study, the concentrations of both *ALDH1A2* and *ALDH1A3* mRNAs were lower in the mesonephroi than in the gonads of either sex, suggesting that in the human the mesonephros may not be capable of secreting large concentrations of RA. Bowles et al (2006) confirmed the synthesis and secretion of RA by the mouse mesonephros at e13.5 and proposed a model whereby

the mesonephric tubules, situated adjacent to the anterior pole of the gonad may be capable of secreting RA into the adjacent gonad, or producing cells that synthesise RA and then that migrate into the gonad. This model agrees with the observation that meiosis is initiated at the cranial end close to the mesonephros (Bendsen et al., 2006; Byskov, 1975)

In our study, incubation of the human mesonephros on the same cell line did not result in activation of the RARE reporter. However mouse mesonephroi recovered at e11.5 and e13.5 failed to induce a quantifiable response from these cells, suggesting that the methods which we used for this assay were not sufficient to induce an appropriate response. For example there may have been slight differences in the procedures used by Bowles et al in either the mechanical dissociation of the mesonephroi or the volume of media used for the cultures. Different mouse strains were also used, in our study C57Bl6 mice were used, while Bowles et al collected mesonephroi from the Quakenbush-Swiss strain. Additionally the cells may not have been as responsive as previously reported (Bowles et al., 2006; Wagner et al., 1992). Ideally the experiments should be repeated again with the same mouse strain as used by Bowles et al, and with new reporter cells.

Another question mark regarding the role of the mesonephros in providing signals that induce meiotic entry in the female through production of RA is prompted by the differences that exist between species in the timings of regression of the mesonephros. In the human, the mesonephros is reported to begin its regression at 10 weeks of gestation (Moritz and Wintour, 1999), at the time when female germ cells are beginning to enter meiosis. The rodent mesonephros persists until relatively late in gestational development, regressing at e15.5 (Vazquez et al., 1998), by which time all oocytes have entered meiosis. Therefore if RA is to influence meiotic entry in the human, it is more likely to be that RA producing cells are located within the human fetal gonad. Although we have demonstrated *ALDH1A2* and *ALDH1A3* enzymes are expressed in the human gonads, with higher levels of expression of

ALDH1A2 in the ovary than the testis, to date we have not been able to demonstrate production of RA, and therefore as yet this hypothesis is unproven.

4.4.4 *CYP26B1* is present in the ovary and testis during the 1st and 2nd trimester

Analysis of the expression of *CYP26B1* in the human fetal gonads revealed that this enzyme was expressed in both the testes and ovaries, and contrary to expectations, mRNA concentrations were higher in the ovary than in the testis during the early second trimester. This is in contrast to the situation in the mouse where expression of *Cyp26b1* is upregulated specifically in the testis at the time when germ cells in the ovary enter meiosis. Additionally in the mouse, *Cyp26b1* transcripts have been found to be specific to the somatic cells of the testis including the Sertoli cells (Bowles et al., 2006; Koubova et al., 2006). Therefore, in the human, the cellular expression of *CYP26B1* must be determined in the ovary and testis. In this study, messenger RNAs for *CYP26A1* and *CYP26C1* could not be detected in either the developing human fetal ovaries or testes and neither of these are expressed in the mouse gonad (Bowles et al., 2006). Studies have shown in cell lines, that stimulation by RA results in an upregulation in both *Cyp26a1* and *Cyp26b1* mRNA expression (White et al., 2000) and that the promoter region of *Cyp26a1* contains a RARE (Loudig et al., 2000). Additionally, Cyp26b1 expression can be upregulated by signalling through RARα (Reijntjes et al., 2005).

4.4.5 *SDMG1* is expressed at high levels in the 2nd trimester human fetal testis

Expression of *SDMG1* mRNA appeared higher in human fetal testis than in the ovary. In the mouse, Sdmg1 is specifically upregulated in the Sertoli cells of embryonic testes at e12.5, where it is localised to the endosomes (Best et al., 2008). It is not expressed in the ovary until postnatal life. Disruption to *Sdmg1* resulted in membrane trafficking defects, and when secretion by the Sertoli cells was inhibited, it resulted in germ cells undergoing male-to-female sex reversal (Best et al., 2008).

In our study, *SDMG1* total mRNA concentrations were highest in the human fetal testis during the 2nd trimester, so upregulation of this gene appears to occur in the human later than in the mouse and interestingly Sdmg1 was expressed in the human fetal ovary at low levels throughout the time points analysed.

4.4.6 Conclusions

The results presented in this chapter have shown for the first time that *STRA8* mRNA expression is restricted to the human fetal ovary, at the time when germ cells are entering meiosis, and that *NANOS2* is expressed at higher levels in the 2nd trimester human fetal testis than the ovary. This is an exciting indication that the mechanisms of meiotic entry and germ cell commitment to their sex-specific fate are at least partially conserved between the mouse and human. RA appears to be important in inducing germ cells to enter meiosis in the mouse, and the novel findings of this chapter that the RARs and RXRs are expressed in the human fetal ovaries and testis suggests that if RA is present it may act to regulate gene expression within the cells of the gonad. Messenger RNA for two of the RA producing enzymes, *ALDH1A2* and *ALDH1A3* were detected in the ovaries and testis and were found at lower levels in the mesonephroi, indicating differences between the mouse and human. Unfortunately, whether either the mesonephroi or gonads produce RA, was not determined. Intriguingly, the results presented here, show that the enzyme responsible for RA break down, *CYP26B1* was found to be expressed in both the ovary and testis, unlike data in the mouse, and given the spatial differences in meiotic progression between the mouse and the human it therefore appears that the model whereby RA induces meiotic entry of germ cells in the mouse is not completely applicable to the developing human gonad.

5 Dissociation and culture of the somatic cells from the human fetal testis

5.1 Introduction

In 1st trimester human fetal testes, germ cells are initially characterised as a uniform OCT4 positive/VASA negative population, but by the 2nd trimester a heterogeneous population exists as germ cells differentiate into OCT4 negative/VASA positive prespermatogonia (Anderson et al., 2007; Gaskell et al., 2004) (Chapter 3). Germ cell maturation takes place in the seminiferous cords in close association with the Sertoli cells, and surrounded by the peritubular myoid cells (PTM) and interstitial cells, including the Leydig cells. These somatic cells are thought to play an important role in regulating the maturation and differentiation of germ cells and in preventing male germ cells from entering meiosis during fetal life, as occurs in females.

In the human, the genital ridges appear in the intermediate mesoderm in the fourth week of gestation, where they form as paired structures on the ventromedial sides of the mesonephroi. Within the centre of the genital ridge coelomic epithelial cells begin to proliferate (Wartenberg, 1981). The Sertoli cells polarise, aggregate together and enclose the germ cells, which migrate into the gonad by the fifth week of gestation (Wartenberg, 1981) (Chapter 1, section 1.3.1.7). The Sertoli cells are themselves enclosed by the PTM cells; these are cells of mesenchymal origin, which express the myofibroblast markers α-smooth muscle actin (α-SMA) and desmin (DES) (Nishino et al., 2001). PTM cells are thought to migrate into the genital ridge from the mesonephros (Martineau et al., 1997) (Chapter 1, section 1.2.3). The recruitment of cells from the mesonephros is male-specific (Merchant-Larios et al., 1993; Capel et al., 1999) and is necessary for the formation of the testis cords (Tilmann and Capel, 1999). Cord formation occurs within only a few hours in the rodent, while in the human, it takes place over a number of weeks, and is not complete until around the 8th week of gestation (Wartenberg, 1981).

The interstitial compartments, situated between the cords contain the fibroblasts, Leydig cells and blood vessels (Brennan and Capel, 2004). Mesonephric cell migration is thought to contribute a number of cells to the interstitium, bringing endothelial cells, perivascular cells and fibroblasts (Buehr et al., 1993; Martineau et al., 1997; Merchant-Larios et al., 1993). Fibroblast cells express the intermediate filament protein vimentin (VIM) (Steinert et al., 1984). In the fetal testis, the fibroblast cells within the interstitium, as well as PTM cells, express the androgen receptor (AR) (Gaskell et al., 2004; Majdic et al., 1995).

Leydig cells are evident from around 9 weeks gestation in the human (Codesal et al., 1990; Ostrer et al., 2007). The primary function of the Leydig cells is the secretion of steroids. Human fetal Leydig cells have been shown to express enzymes required for steroid synthesis such as, side chain cleavage p450 (SCCP450) and 3-β-hydroxysteroid dehydrogenase (3βHSD**)** throughout the 2nd trimester (Murray et al., 2000). The development of the Leydig cells is at least partly dependent on the secretion of paracrine signalling molecules from the Sertoli cells. The most well characterised Sertoli-cell derived signalling molecule is desert hedgehog (Dhh) this molecule acts upon the patched (Ptch) receptor expressed by the Leydig cells, and may lead to the upregulation of steroidogenic enzymes such as SCCp450 (Clark et al., 2000; Yao et al., 2002)**.**

In most mammals, the gene which initiates the cascade resulting in the development of the testis is located on the Y chromosome, referred to as the Sex-determining region of the Y chromosome (*SRY)* (Koopman et al., 1990; Lovell-Badge and Robertson, 1990). *SRY* encodes a putative transcription factor that is expressed in cells within the interior of the genital ridge between e10.5 and e12.5 in the mouse (Hacker et al., 1995; Bullejos et al., 2001; Albrecht and Eicher, 2001). The primary function of SRY is to trigger the differentiation of the Sertoli cells (Palmer and Burgoyne, 1991) (Chapter 1, section 1.2.2.1). In the human testis, *SRY* mRNA is first detectable at about 6 weeks gestation (Hanley et al., 2000; Poulat et al., 1995),

and unlike the transient expression observed in the mouse, mRNA is still detectable in the human testis throughout fetal life (Hanley et al., 2000) and even into adulthood (Salas-Cortes et al., 1999).

The only identified target of SRY thus far has been the HMG-box containing transcription factor SOX9 (Bishop et al., 2000; Vidal et al., 2001) (Chapter 1, section 1.2.2.2). Once gene expression is initiated, *Sox9* continues to be expressed in the testis throughout development (Morais da Silva et al., 1996). In the human *SOX9* mRNA is expressed in the human male genital ridge, where its expression pattern closely parallels that of *SRY*, with transcripts detectable as early as 44 days post ovulation (Hanley et al., 2000), and persisting throughout gestation in the testes.

Also implicated in the regulation of *Sox9* expression is the transcription factor Wilms Tumour 1 (WT1). When *WT1* is mutated, a host of genitourinary abnormalities occur, including sex reversal (Barbaux et al., 1997; Pelletier et al., 1991). Mutations in *Wt1* in the mouse result in a failure of the genital ridge to thicken at e10.5, and as a consequence no gonad forms (Kreidberg et al., 1993). Sertoli cell specific ablation of *Wt1* was shown to lead to a downregulation of Sox9 at e14.5 (Gao et al., 2006). WT1 has been shown to be highly expressed in the coelomic epithelium of the human at 6.5 weeks and after formation of the cords its expression becomes restricted to the Sertoli cells (de Santa Barbara et al., 2000; Hanley et al., 1999).

The fetal Sertoli cells secrete the glycoprotein anti-mullerian hormone (AMH), which plays a critical role in stimulating the regression of the Mullerian ducts in the male (Jost et al., 1953), and may have other important roles within the testis (Ross et al., 2003). Expression of *Amh* is initiated in the developing Sertoli cells of the mouse at e12.5 (Munsterberg and Lovell-Badge, 1991) and in the human, AMH has been shown to be expressed in the fetal testis during the 7th week of gestation, just prior to cord formation (de Santa Barbara et al., 2000; Gaskell et al., 2004). AMH is expressed throughout fetal development at high levels, and expression is maintained after birth and is downregulated at puberty (Tran et al., 1987; Rey et al., 2003).

Another important transcriptional regulator important for testis differentiation is GATA4 (Tevosian et al., 2002). GATA4 belongs to the GATA family of transcriptional regulators (Arceci et al., 1993) and is expressed in the somatic cells of the gonad where it has been localised to the Sertoli cells, Leydig cells and fibroblasts (Viger et al., 1998). Mouse mutants of *Gata4*, and a knock-in mutation affecting the ability of Gata4 to interact with its co-factor Fog2, both display an identical phenotype, exhibiting decreased expression of *Sry*, decreased cellular proliferation and abnormal Sertoli cells and Leydig cell development (Tevosian et al., 2002). A recent study suggests that in the absence of *Gata4*, Leydig cells cannot form (Bielinska et al., 2007).

SF1 is an orphan nuclear receptor which acts to regulate the transcription of a number of target genes that control of the gonadal and adrenal development. *Sf1* homozygous mutant mice display gonadal and adrenal agenesis (Lala et al., 1992; Luo et al., 1994). At e11.5, *Sf1* expression is found in both the pre-Sertoli cells and the pre-Leydig cell population, but thereafter *Sf1* expression decreases in Sertoli cells and increases in differentiating Leydig cells (Hatano et al., 1996). This pattern of expression appears to be mirrored in the human, where prior to formation of the cords, *SF1* localises primarily to the developing Sertoli cells, while after the cords form *SF1* is more abundant in the Leydig cells (Hanley et al., 1999; Majdic and Saunders, 1996).

A number of paracrine signalling systems are thought to operate in the testis, one important group of signalling molecules are the activins. Activins are members of the TGF-β family; they exist as dimers of 2 homologous protein B subunits, with homodimers of the activin subunit forming activin A ($\beta_A\beta_A$) or activin B ($\beta_B\beta_B$). Activins mediate their effect on cell function by binding to the type II receptor serine kinases, ACTRIIA or ACTRIIB, these then recruit and phosphorylate the type I activin receptor-like kinases ALK2 or ALK4, which in turn activate SMAD proteins (Mathews, 1994). Phosphorylation of SMAD2 and SMAD3 enables them to

dimerize with SMAD4, and the SMAD complex then migrates to the nucleus where it induces transcriptional responses (Massague, 1998; Massague and Chen, 2000) (Chapter 1, section 1.4.1). Sertoli cells, PTM cells and Leydig cells within the human fetal testis all have the potential to produce activins (Majdic et al., 1997; Anderson et al., 2002). In the human fetal testis, ACTRIIA has been immunolocalised to the interstitium, as well as the gonocytes and some Sertoli cells, while ACTRIIB was found to be expressed in the interstitium, the gonocytes and the Sertoli cells, as well as the PTM (Anderson et al., 2002). In the human fetal ovary, activins may have an important role in germ cell survival (Martins da Silva et al., 2004), where it appears to act upon the somatic cell (Coutts et al., 2007a), and in the immature rat testis, activin A has been shown to stimulate proliferation of Sertoli cells (Boitani et al., 1995).

Some progress has been made in establishing explant cultures of human fetal testes (Coutts et al., 2007b; Hallmark et al., 2007; Robinson et al., 2003). Culture of isolated populations of somatic cells from postnatal human testis has been achieved, but with a limited success (Berensztein et al., 2000; Berensztein et al., 1992; Rivarola et al., 1995).

5.1.1 Aims of chapter

This study aimed to establish cultures of testicular somatic cells that maintain a differentiated phenotype in order to provide a model system to further understand gene expression in the 2nd trimester human fetal testis. Cell-type specific expression of putative marker proteins was investigated using immunohistochemistry on fixed tissue sections. Isolated populations of human somatic cells were maintained *in vitro* and gene expression was then analysed in these cell types. Additional experiments aiming to optimise the dissociation procedure and culture conditions were also performed.

5.2 Materials and methods

5.2.1 Collection of human fetal testes

Human fetal testes were obtained following termination of pregnancy as outlined in Section 2.1

5.2.1.1 Immunohistochemistry

Immunohistochemistry on fixed tissue sections of testis was performed and is detailed in section 2.2. Information regarding the primary antibodies used is listed in (Table 5-1).

Table 5-1 Summary of primary antibodies used for immunohistochemistry

Antigen	Source	Species	Dilution	Citrate Retrieval
3βHSD	Gift from Professor Ian Mason. University of Edinburgh	Rabbit	1:1000	No
AMH	Santa cruz	Goat	1:500	No
AR	Santa Cruz	Rabbit	1:200	Yes
DES	Dako	Mouse	1:200	Yes
GATA4	Santa Cruz	Goat	1:100	Yes
SCCp450	Chemicon	Rabbit	1:200	No
SOX9	Chemicon	Rabbit	1:200	Yes
WT1	Dako	Mouse	1:50	Yes
α-SMA	Sigma	Mouse	1:200	No

5.2.1.2 Fluorescent immunohistochemistry

Fluorescent immunohistochemistry on tissue sections was performed following the procedure outlined in section 2.3.10. For double staining of AR and SMA, AR was used at a dilution of 1:50. Goat anti-rabbit biotinylated followed by streptavidin 546 was used for detection of AR. SMA was used at a dilution of 1:100, followed by goat anti-mouse alexa 488.

5.2.2 Dissociation of human fetal testes

Testes were removed from male fetuses and placed in 4mls of Hanks Buffered Salt Solution (HBSS) (Gibco). Under a dissection microscope, a section of testis was removed and snap frozen for extraction of RNA or protein (time 0). The remaining testis was carefully pulled apart using a 40mm needle. In a class II tissue culture hood, 500µl of collagenase type IV (Sigma) (0.01g in 1 ml HBSS) was added to the 4mls of HBSS containing the tissue and incubated for 15 minutes, at 34^{o}C on a roller. Thereafter, 50µl of DNase 1 (Sigma) (diluted 0.007g in 1ml HBSS) was added to the solution and the mixture was incubated for a further 5 minutes. The resulting cell suspension was centrifuged for 5 minutes at 800 rpm, the supernatant was discarded, and the cell pellet was re-suspended in 4mls of HBSS (Gibco) to wash. The washing step was repeated twice.

In some cases, 1ml of trypsin/EDTA (Sigma) was then added to the cell suspension and incubated for 10 minutes. Once a single cell suspension was obtained, 0.5ml of fetal calf serum was added to prevent any further action of trypsin. The cells were filtered through a 70µm mesh filter and centrifuged at 800 rpm for 5 minutes, they then were resuspended in 1ml of media, and a cell count performed.

5.2.3 Culture of human fetal somatic cells

Cells were plated onto 0.01% gelatin (Sigma) coated plates, T25 flasks or chamber wells. The recipe of the medium used is listed in section 2.7. Media was supplemented with dihydrotestosterone (DHT) (Sigma) at a concentration of $1x10^{-8}$M. Cells were passaged when confluent, normally every 4-6 days. Passaging was performed by either using trypsin/EDTA or dispase (Sigma) (2 mg/ml). Medium was changed every 2nd day. For activin experiments, activin A (R&D systems) was used at a concentration of 10 ng/ml (diluted in HBSS).

5.2.4 RNA Extraction

Details of the extraction of RNA from tissue and cells are as described in section 2.4.

5.2.5 Preparation of cDNA using random hexamers

cDNA was prepared as outlined in section 2.5.2.1.

5.2.6 TaqMan analysis

TaqMan quantitative RTPCR was performed using the Roche Universal Probe Library. Details of the TaqMan reaction and method of analysis are described in section 2.5. Primers used, along with the corresponding probe number are listed in Table 5-2. Data was expressed relative to positive control (pooled RNA from 3 whole human fetal testes).

5.2.7 Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by a Bonferonni multiple comparison test.

Table 5-2 Summary of primers and probes used for TaqMan reaction

Gene name	5' sequence	3'sequence	Probe number
3βHSD	cggaccagaattgagagagg	gaatggctcatccagaatgtc	11
ACTR IIB	tgtcaagatcttcccactcca	catgccaggtgtgctgaa	53
ACTRIIA	aaagcccagttgcttaacga	tgccatgactgtttgtcctg	5
ALK4	atattgggagattgctcgaaga	ggcagctgatattcttcatgg	65
ALK2	catgaatttggcttttggaga	ctttggcagtgtgtgacgctta	1
AMH	cgcctggtggtcctacac	gaacctcagcgagggtgtt	69
AR	gccttgctctctagcctcaa	gtcgtccacgtgtaagttgc	14
DES	ggagattgccacctaccg	ggtctggatggggagattg	55
DHH	gcaacaagtatgggttgctg	cggaccgcagtgagtta	86
GATA4	ggaagcccaagaacctgaat	gggaggaaggctctcactg	17
OCT4	gtggagagcaactccgatg	tgcagagctttgatgtcctg	78
SCCp450	aggaggggtggacacgac	ttgcgtgccatctcataca	59
SF1	gcaggtgcatggtcttcaa	agttctgcagcagcgtcat	10
SMA	ctgttccagccatccttcat	tcatgatgctgttgtaggtggt	58
SMAD2	gcttctctgaacaaaccaggtc	atgtggcaatccttttcgat	80
SOX9	gtacccgcacttgcacaac	tctcgctctcgttcagaagtc	61
SRY	gcttactgaagccgaaaaaatg	tctctgtgcatggcctgtaa	79
VASA	cgccaaaacccttatgttcag	aaaaactctgcagccaacctt	6
VIM	gaccagctaaccaacgacaaa	gaagcatctcctcctgcaat	39
WT1	ggagccaccttaaagggagt	gcccttctgtccatttcact	3

5.2.8 Immunohistochemistry on cultured cells

Cultured cells were seeded onto polyL lysine-coated CC2 chamber slides (Lab-Tek, Nalge Nunc International, Roskilde, Denmark). Cells were either cultured for 3 hours to allow attachment onto the slide or grown until confluent.

For DAB immunohistochemistry, cells were fixed in ice cold methanol for 10 minutes at -20°C. Cells were blocked in TBS containing 20% normal serum and 5% BSA, followed by the avidin-biotin blocking kit (refer to section 2.3.6), after which primary antibodies were added and incubated overnight at 4°C. After 2x5 minute washes in TBS, cells were incubated with appropriate secondary antibodies for 30 minutes. Cells were then washed in TBS and incubated with Streptavidin-HRP for 30 minutes, followed by DAB (refer to section 2.3.9) and counterstained with haematoxylin. Primary antibodies used and their corresponding secondary antibodies are listed in Table 5-3.

Table 5-3 Primary antibodies used for immunohistochemistry on cultured cells

Antibody	**Supplier**	**Dilution**	**Secondary antibody**
DES	Dako	1:200	GAM-b
VIM	AbCam	1:300	GAR-b

For fluorescent immunohistochemistry, cells were fixed in Modified Davidson's Fixative for 10 minutes at room temperature, followed by 2x5 minute washes in PBS. Cells were blocked in PBS containing 20% normal serum and 5% BSA, and then with the avidin-biotin blocking kit. Primary antibodies were added and incubated overnight at 4°C and are listed in Table 5-4. After 2x5 minute washes in PBS, cells were incubated with appropriate secondary antibodies. Cells were then washed and the appropriate fluorescent molecules were added, diluted in PBS, following 2x5 minute washes, Dapi (Sigma, diluted 1 in 1000) was incubated with the cells for 10 minutes. Slides were mounted in permaflour.

Table 5-4 Primary antibodies used for fluorescent immunohistochemistry on cultured cells

Antibody	Dilution	Secondary antibody	Flourescent Label
GATA4	1:200	RAG-b	Streptavidin-546
SCCp450	1:100	RAG-b	Streptavidin-546
SMA	1:200	GAM-b	Streptavidin-546
SOX9	1:1000	GAR-b	Streptavidin-546

5.2.9 Western blotting

Western blotting was performed on total protein extracted from human fetal testes obtained during the 1st trimester and 2nd trimesters. Briefly, testes were halved and lysed in RIPA buffer (Section 2.6.1) and total concentration of protein was quantified using a Biorad protein assay (Section 2.6.2). Full details of the Western blotting technique are outlined in Section 2.6. Information regarding the primary antibodies used is listed in Table 5-5

Table 5-5 Details of primary antibodies used for Western Blotting

Antigen	Host Species	Source	Dilution
SOX9	Rabbit	Chemicon	1:500
GATA4	Goat	Santa Cruz	1:300
SMA	Mouse	Sigma	1:500
AR	Rabbit	Santa Cruz	1:300
β-TUBULIN	Rabbit	Santa Cruz	1:1000
β-TUBULIN	Mouse	Sigma	1:1000

5.3 Results

5.3.1 Immunohistochemical staining for putative Sertoli cell specific proteins in human fetal testes

Human fetal testis were immunopositive for SOX9 throughout the 2nd trimester and the protein was localised to the nuclei of all Sertoli cells (Figure 5-1 a and b). AMH was immunolocalised to the cytoplasm of Sertoli cells throughout the 2nd trimester (Figure 5-1 c and d).

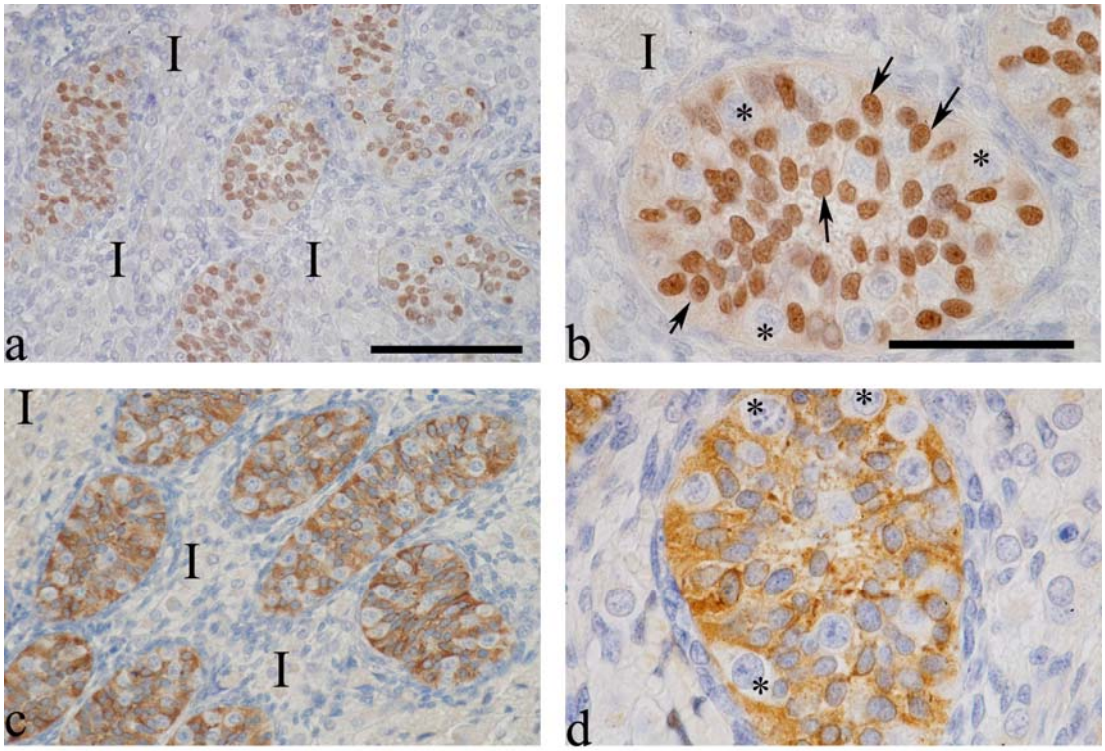


Figure 5-1 Immunoexpression of Sertoli cell markers in 2nd trimester human fetal testes. SOX9 staining of human fetal testis at a) 16 weeks b) 17 weeks and AMH immunostaining of c) 17 week and d) 15 week human fetal testis. I labels the interstitium, arrows point to immunopositive Sertoli cells and asterisks indicated immunonegative germ cells. For a and c, bar=100µm, for b and d, bar=50µm.

5.3.2 Immunohistochemical staining of WT1 and GATA4 in the human fetal testis

Sertoli cells were also immunopositive for WT1, which was localised to the Sertoli cell nuclei of 2nd trimester testis as well as some unidentified cells within the interstitium (Figure 5-2 a and b). GATA4 was immunolocalised to the nuclei of the Sertoli cells, PTM cells, and some Leydig cells and interstitial fibroblasts (Figure 5-2 c and d).

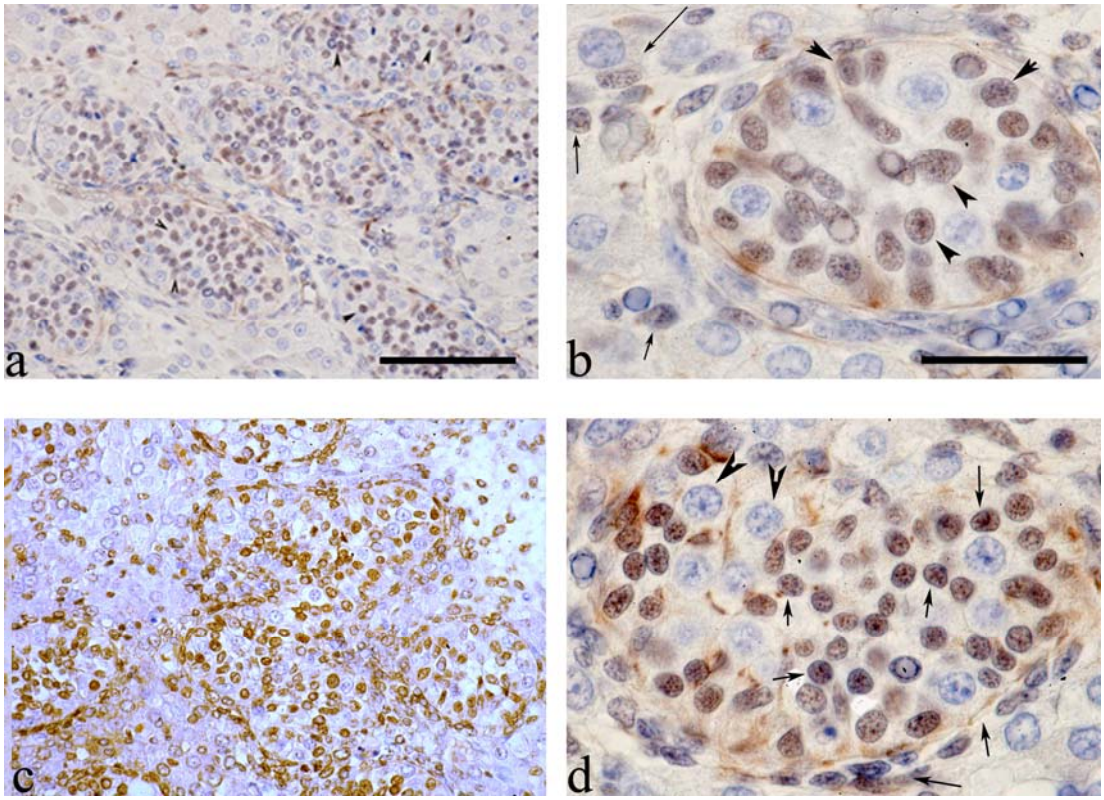


Figure 5-2 Immunostaining of WT1 in 2nd trimester human fetal testis at a) 15 weeks and b) 19 weeks. Immunostaining for GATA4 at c) 17 weeks and d) 19 weeks. For a and b arrows show immunopositive interstitial cells, while arrowheads point to positive Sertoli cell nuclei. For c and d arrows point to immunopositive Sertoli and PTM cells, arrowheads label GATA4 negative germ cells. Bar=100 µm for a and c and 50µm for b and d.

5.3.3 Immunohistochemical staining of Leydig cell markers in human fetal testis

3βHSD was immunolocalised exclusively to cells within the interstitium of 2nd trimester human fetal testis, in cells presumed to be Leydig cells, where staining was confined to the cytoplasm (Figure 5-3, and b). SCCP450 was also immunolocalised to these presumptive Leydig cells, with a non-uniform staining pattern consistent with its localisation to the microsomes (Figure 5-3, c and d).

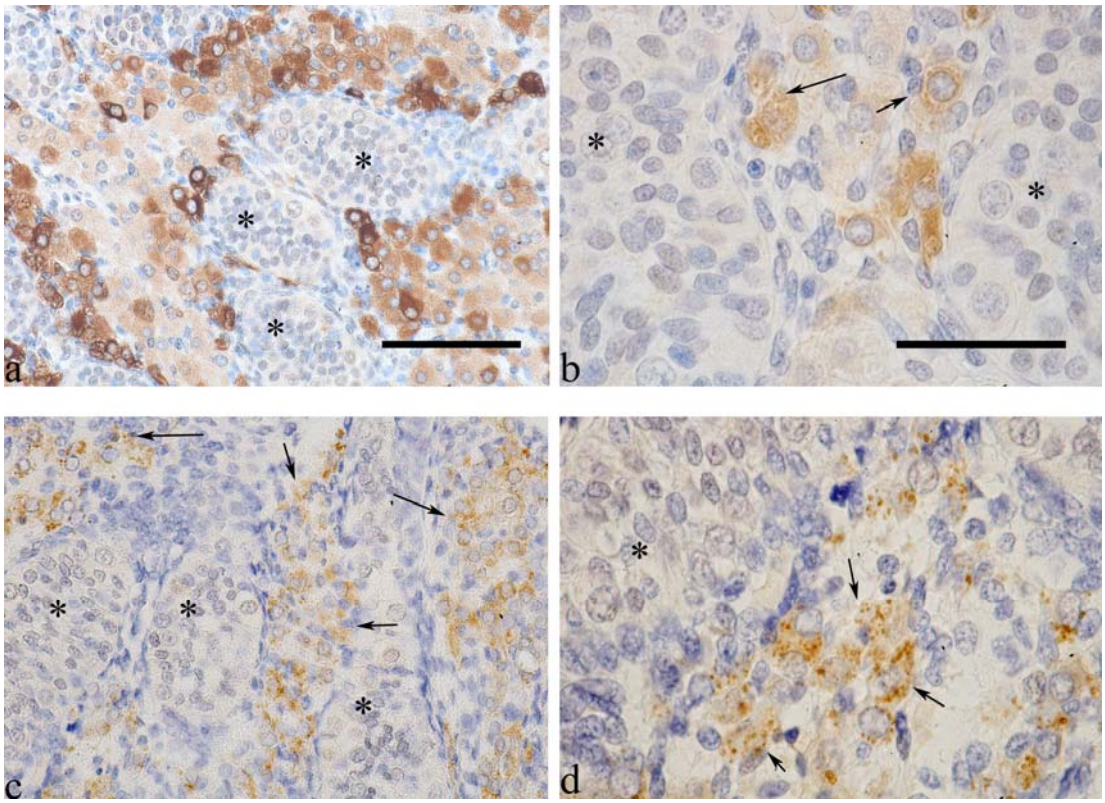


Figure 5-3 Immunostaining of Leydig cell markers in 2nd trimester human fetal testis. a) 3βHSD staining of 17 week human fetal testis b) 3βHSD staining of 16 week human fetal testis c)SCCp450 immunostaining of 16 week human fetal testis d) 15 week human fetal testis stained for SCCp450. Asterisks label the testicular cords and arrows point to immunopositive Leydig cells. For a and c, bar=100μm, for b and d, bar=50μm.

5.3.4 Immunohistochemical staining of AR in the human fetal testis

AR was immunolocalised to the nuclei of small interstitial cells (presumptive fibroblasts) and cells adjacent to the seminiferous cords i.e the PTM cells (Figure 5-4.). Note that both Sertoli cells and germ cells were immunonegative.

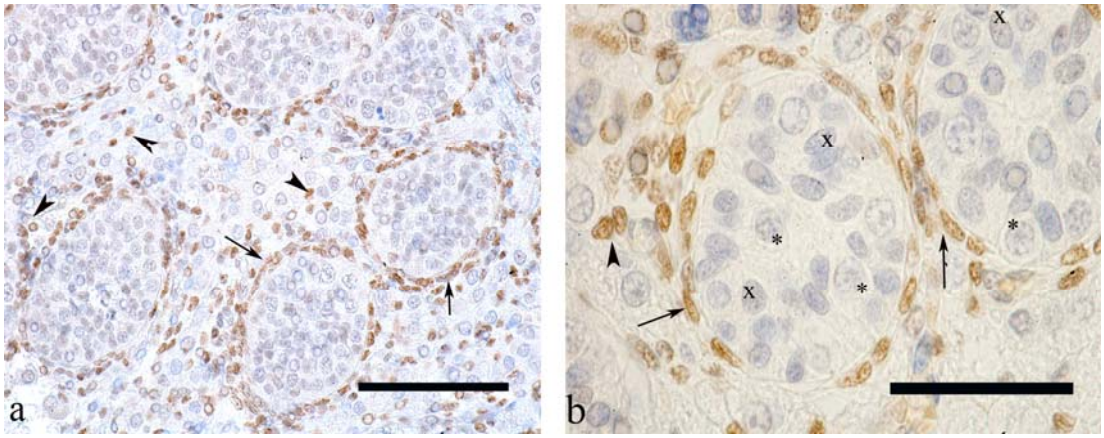


Figure 5-4 Immunostaining of AR in 2nd trimester human fetal testis at a) 16 weeks and b) 18 weeks. Cells labelled with asterisks indicate AR immunonegative germ cells, while immunonegative Sertoli cells are labelled with a cross. Arrows show immunopositive PTM cells, while arrowheads point to interstitial cells immunopositive for AR. Bar=100 µm for a and 50µm for b.

5.3.5 Immunohistochemical staining of PTM cells markers in the human fetal testis

Both SMA and DES were immunolocalised to the cytoplasm of elongated flat cells found surrounding the testicular cords, identifiable as the PTM cells (Figure 5-5). Both SMA and DES were also immunolocalised to cells surrounding blood vessels (Figure 5-5). DES immunopositive staining was detected in many more interstitial cells than SMA staining, the identity of these cells was not explored further, but they are likely to be fibroblasts (Figure 5-5 c and d).

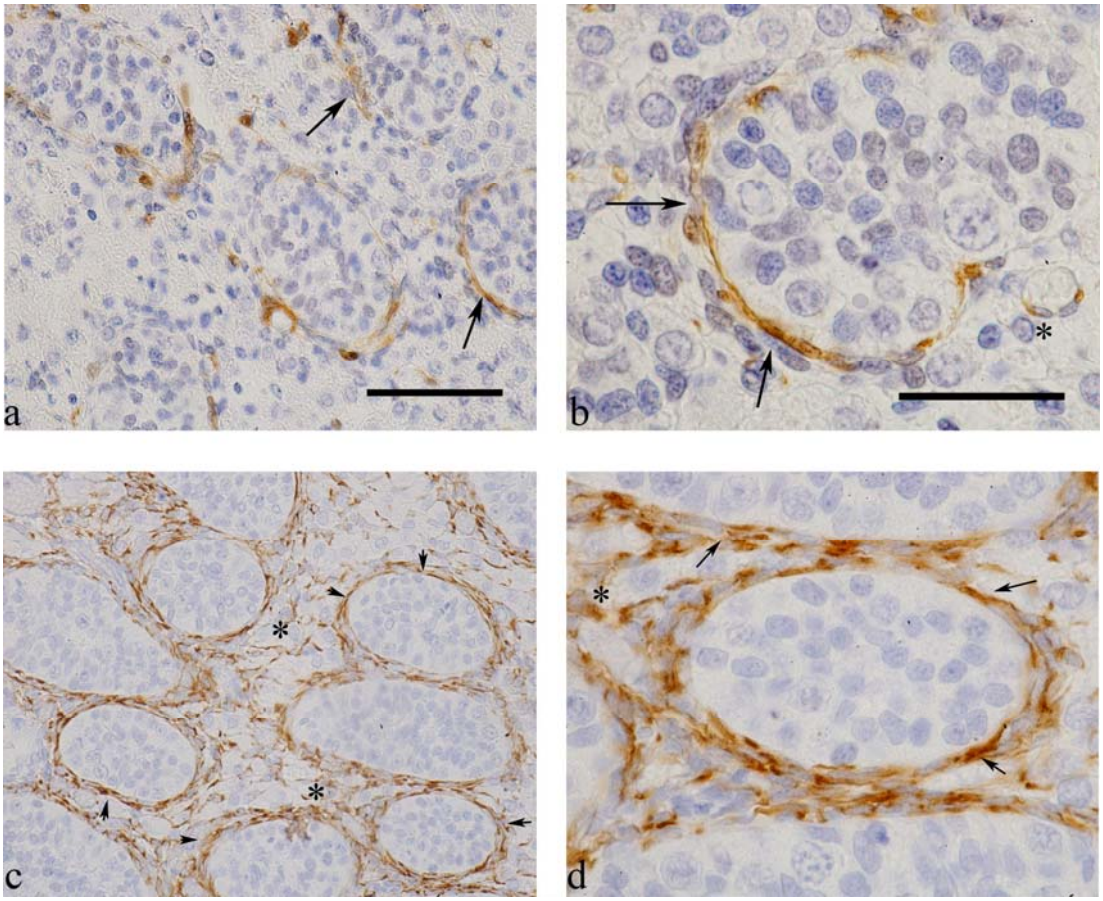


Figure 5-5 Immunostaining of PTM markers in human fetal testis. a) and b) SMA immunostaining of 16 week human fetal testes; c) and d) immunostaining of DES in 16 week human fetal testes. Arrows point to immunopositive PTM cells and asterisks show DES immunopositive interstitial cells. Bar=100μm, for b and d, bar=50μm.

5.3.6 Immunohistochemical colocalisation of AR and SMA in human fetal testis

In order to confirm that some of the AR-positive interstitial cells were PTM cells, sections were co-stained with antibodies directed against AR and SMA. In agreement with the results of the single staining (Figure 5-5), SMA was localised to the cytoplasm of cells surrounding the testis cords and the blood vessels (Figure 5-6, green). AR was detected in the nuclei of multiple cells within the interstitium (Figure 5-6, red nuclei), and PTM cells were identified as AR positive/SMA positive (Figure 5-6b, arrowheads).

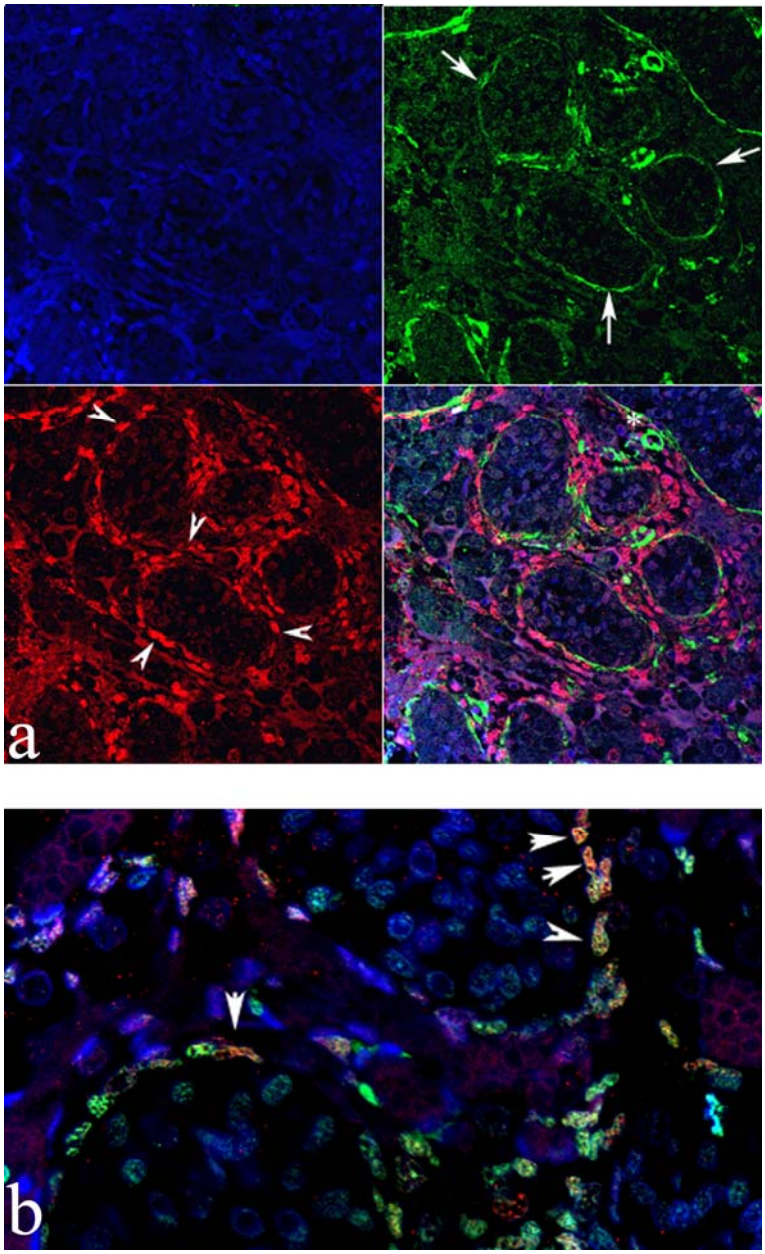


Figure 5-6 Fluorescent immunohistochemistry showing co-localisation of SMA (green) and AR (red) in 2nd trimester human fetal testis at a) 18 weeks b) 16 weeks. In a arrowheads show AR positive cells and arrows label cells that are immunopositive for SMA, in b arrows point to cell positive for both AR and SMA

5.3.7 Morphology of dissociated and cultured 2nd trimester human fetal testis

Once dissociated and placed in the culture dishes, the cells adhered to the gelatin-coated plates quickly and began to divide within a few days. The morphology of the cells in the cultures was not uniform, with smaller cells closely adherent to one another, as well as rounder cells and cells which appeared long and flat. Cells were confluent normally within 4-6 days, although the time until confluency was reached decreased as the number of passages increased. As the passage number increased the number of long flat cells appeared to increase (Figure 5-7). By passage 5 the morphology of the cells was less mixed, with more cells that were long and flat,

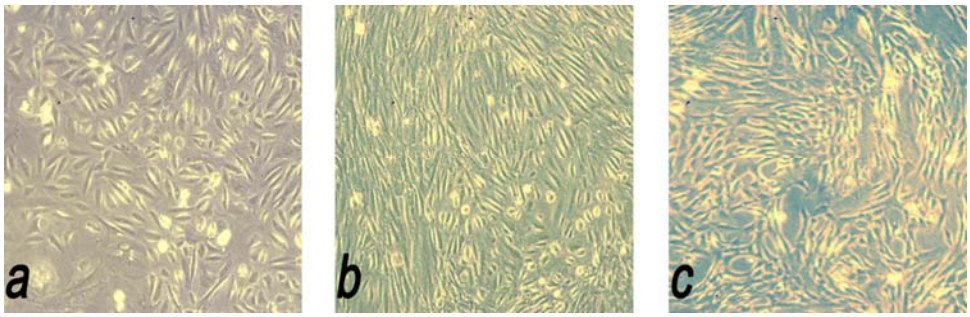


Figure 5-7 Images of cells obtained from a 14 week fetus a) passage 1 b) passage 3 c) passage 5

5.3.8 Expression of germ cell markers in cultures of dissociated testicular cells

TaqMan qRTPCR analysis of cultures using primers specific for the gonocytes specific marker, *OCT4* (Chapter 3) failed to detect the expression of the mRNA even in passage 1 cultures (Figure 5-8a). Likewise *VASA* mRNA, a marker of the prespermatogonial cells (Chapter 3), was not detected in the cultures (Figure 5-8b). This data was taken as evidence that none of the adherent cells in the cultures were germ cell in origin, and therefore for the rest of this chapter, these cultures will be referred to as ‘somatic cell cultures’.

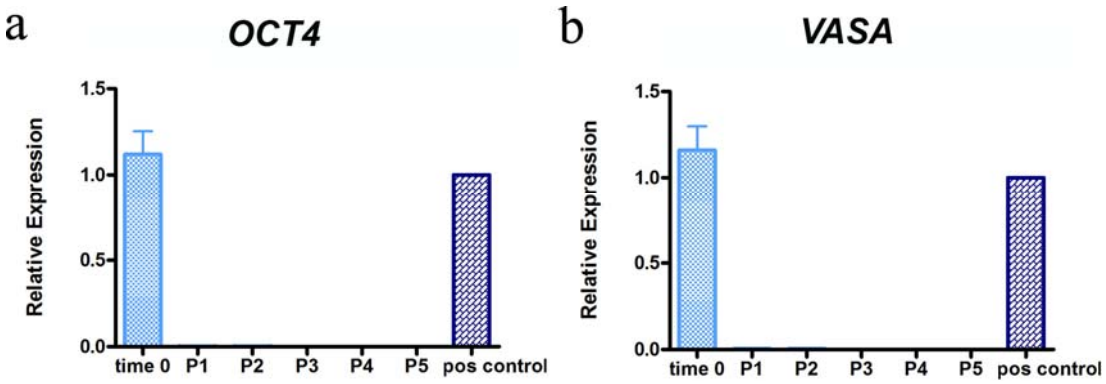


Figure 5-8 TaqMan analysis of germ cell markers on cultured human fetal testicular cells at time 0 (section of fetal testis taken prior to dissociation) and p1-p5, relative to positive control for a) OCT4 and b) VASA. Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

5.3.9 Expression of Sertoli cell markers in somatic cell cultures

Although *AMH* and *DHH* mRNAs were detected in the testes prior to culture, after dissociation the mRNAs were not detected in the cultured cells. Expression of *AMH* was undetectable at P1 (Figure 5-9a) and was significantly reduced compared with time 0 ($P<0.0001$). *DHH* mRNA was also significantly reduced by P1 ($P<0.0001$) (Figure 5-9b).

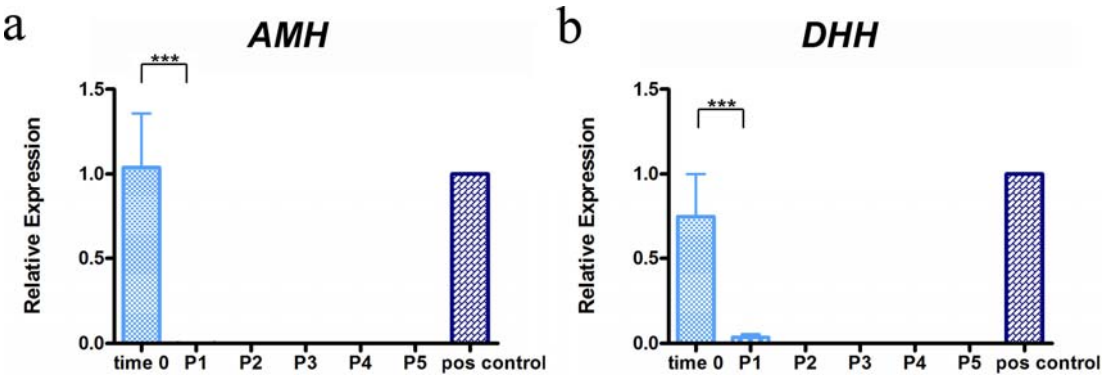


Figure 5-9 Expression of a) AMH and b) DHH mRNA for time 0, p1-5 in dissociated and cultured human fetal testis, relative to positive control (N=4; 15, 17, 2x18 week). Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

In contrast of *SRY* mRNA was detectable both in the 2nd trimester human fetal gonads prior to culture and in the dissociated cells throughout the culture period (Figure 5-10a). *SOX9* mRNA expression was also maintained throughout the culture period (Figure 5-10b). The expression of *SRY* and *SOX9* mRNAs decreased in parallel with each other with increasing passage number. There were no statistically significant changes in *SOX9* and *SRY* throughout the culture period.

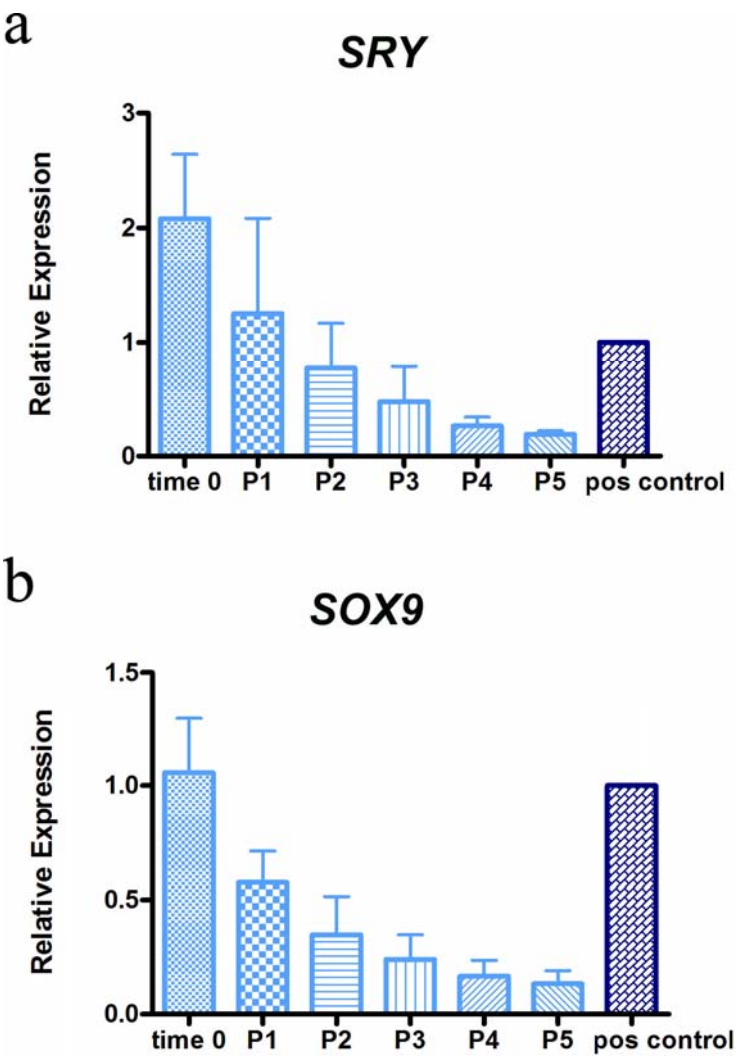


Figure 5-10 mRNA expression for a) SRY b) SOX9 at time 0 and p1-p5, relative to positive control (N=4; 15, 17, 2x18 week) . Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

5.3.10 Expression of *WT1*, *GATA4* and *SF1* mRNA in human fetal somatic cell cultures.

Expression of *WT1* mRNA was also detected in cultures up to and including passage 5 although it decreased as the passage number increased (Figure 5-10c). In contrast, expression of *GATA4* mRNA was maintained throughout the culture period (Figure 5-11a), as was *SF1* (Figure 5-11b), although the total amount of *SF1* mRNA relative to total mRNA appeared to decline as the number of passages increased, while the

expression of *GATA4* appeared to be slightly more constant, even after repeated passage of the cells.

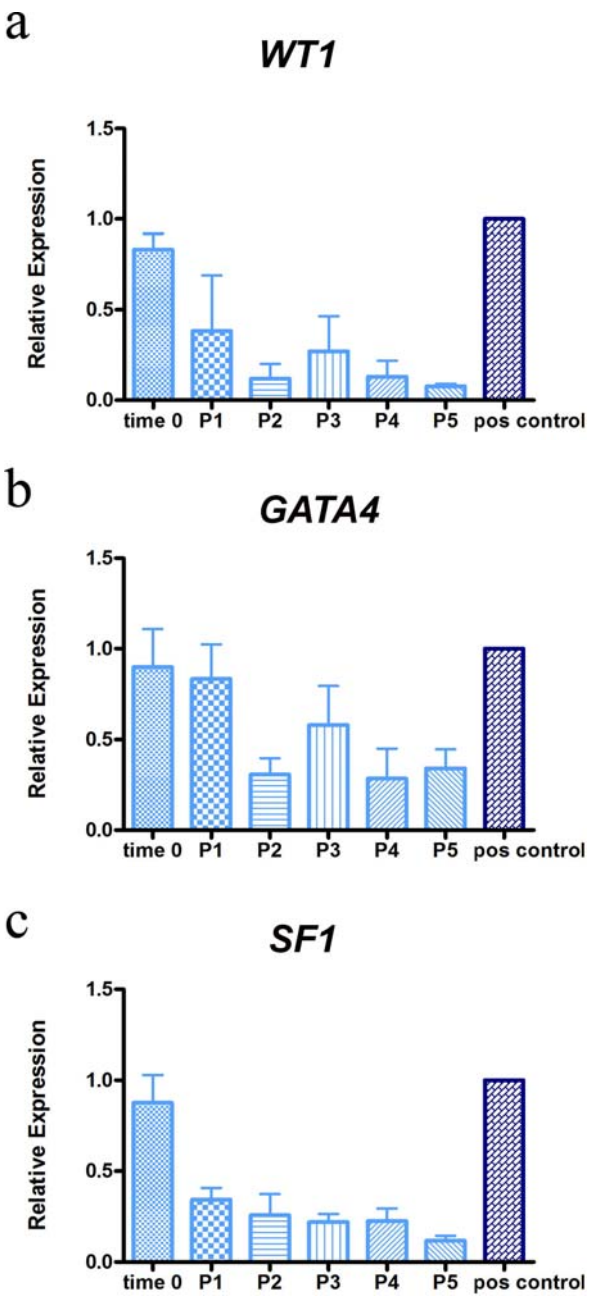


Figure 5-11 a) WT1, b) GATA4, c) SF1 mRNA expression for time 0, p1-p5. All results are given relative to positive control (N=4; 15, 17, 2x18 week). Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

5.3.11 Expression of Leydig cell markers in human fetal somatic cell cultures

In order to determine whether any Leydig cells were present in the somatic cell cultures, qRTPCR analysis was carried out to determine the expression of two Leydig cell specific enzymes, *SCCp450* and *3βHSD* (Figure 5-12). mRNA for both enzymes were detected at passage 1 but then rapidly declined. SCCP450 mRNA levels were significantly reduced at P1 compared with time 0 ($P<0.05$), *3βHSD* was also significantly reduced by P1 ($P<0.01$). Expression of *3βHSD* appeared to be maintained slightly longer than *SCCp450*.

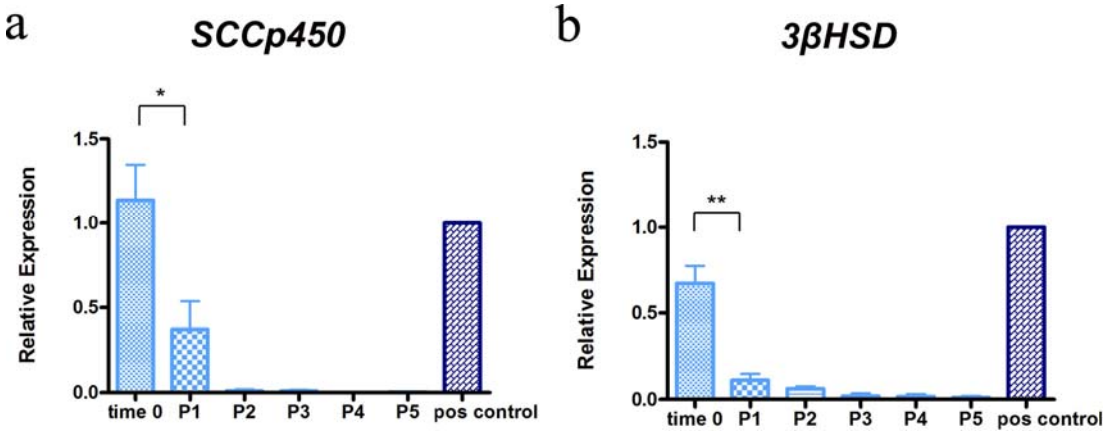


Figure 5-12 3βHSD and SCCp450 mRNAs expression for time 0, p1-p5, relative to positive control (N=4; 15, 17, 2x18 week). Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

5.3.12 Expression of PTM/fibroblast markers in human fetal somatic cell cultures

As the number of passages increased, total expression of *α-SMA* mRNA appeared to increase compared to total concentrations in extracts from intact testes (Figure 5-13a). *α-SMA* mRNA expression was significantly increased at P5 compared with P1 ($P<0.05$). Expression of *DES* mRNA was also higher in the dissociated somatic cell cultures compared with the expression at time 0 ($P<0.05$) (Figure 5-13b). Total

VIM mRNA was similar to time 0 at passage 1 but thereafter increased as a population of total mRNA (Figure 5-13c).

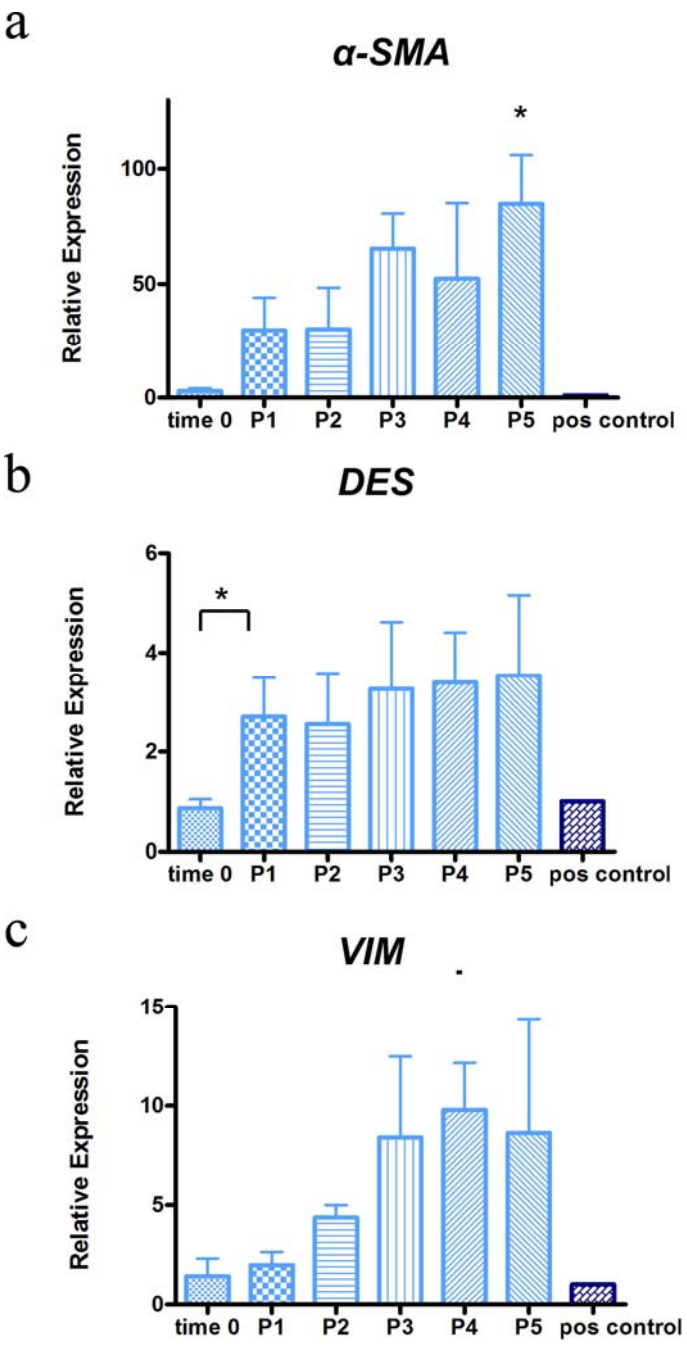


Figure 5-13 α-SMA, DES and VIM mRNA expression for time 0, p1-p5, relative to positive control (N=4; 15, 17, 2x18 week). Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

5.3.13 *AR* mRNA expression in human fetal somatic cell cultures

Throughout the culture period mRNA for the *AR* was detectable, although as a population of total mRNA, it did decline with increasing passage (Figure 5-14).

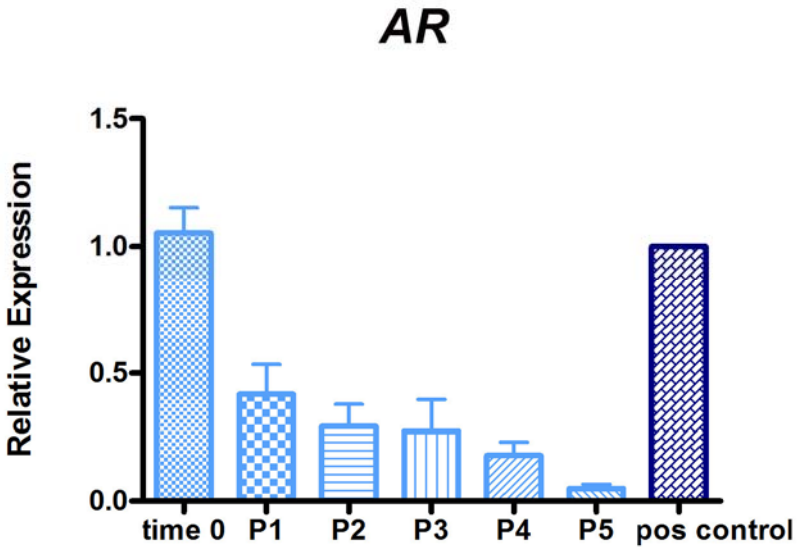


Figure 5-14 AR mRNA expression for time 0, p1-p5, relative to positive control (N=4; 15, 17, 2x18 week). Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

5.3.14 Activin receptor mRNA expression in human fetal somatic cell cultures

Expression of mRNAs encoding the type II activin receptors *ACTRIIA* and *ACTRIIB* was detectable in cells throughout all five passages (Figure 5-15a and b). The mRNAs for type I activin receptors, *ALK2* and *ALK4* were also expressed at concentrations that roughly paralleled those in the intact testis (time 0) and were sustained throughout the culture period (Figure 5-15c and d).

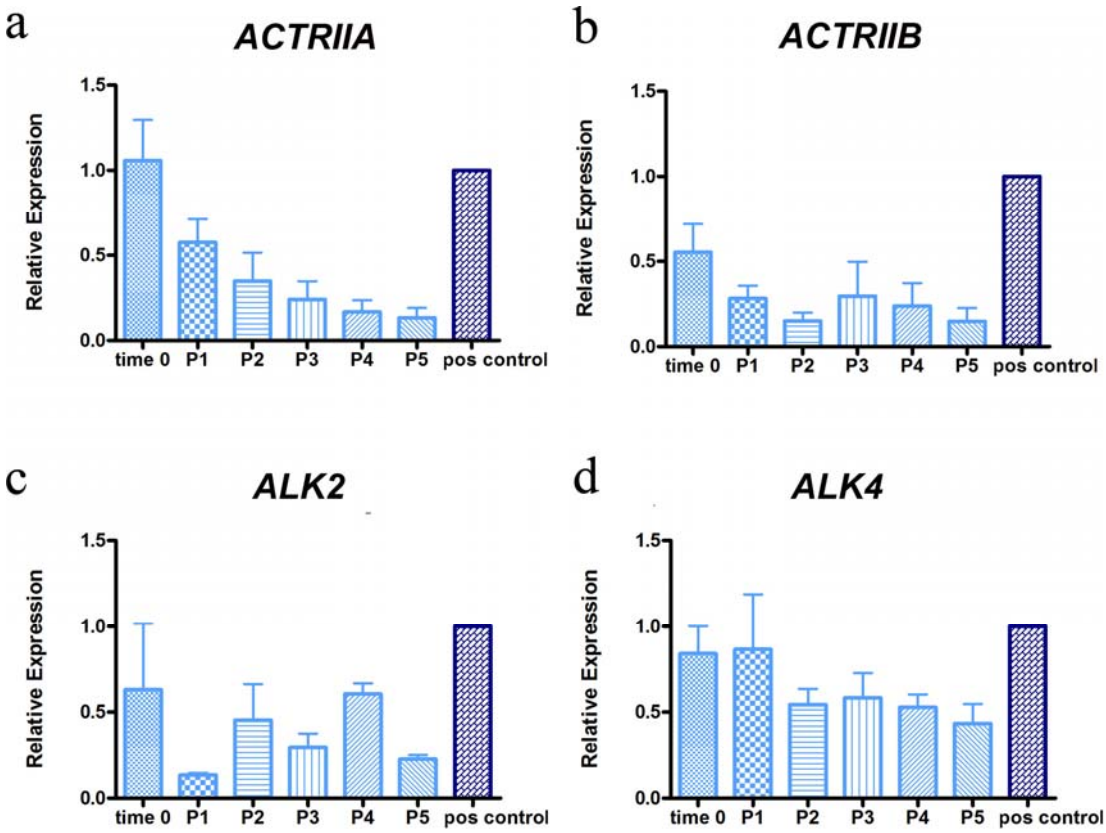


Figure 5-15 Expression of activin receptor mRNA for time 0 and p1-p5. a) ACTRIIA b) ACTR IIB c) ALK2 d) ALK4 (N=4; 15, 17, 2x18 week). Taqman was performed in triplicate for 4 independent cultures from fetuses obtained at 15, 17 and 2x18 weeks. Mean ± sem

5.3.15 Western analysis of human fetal somatic cell cultures

Western analysis was used to detect expression of SOX9 (64kDa), GATA4 (46 kDa), α-SMA (42kDa) and AR (110kDa) protein (Figure 5-16). β-TUBULIN (51kDa) was used as the loading control, for all N=1. SOX9 and GATA4 protein expression was maintained throughout the cultures. SMA protein was also present throughout the cultures, although total protein levels did not appear to show the gradual increase found with the mRNA data.

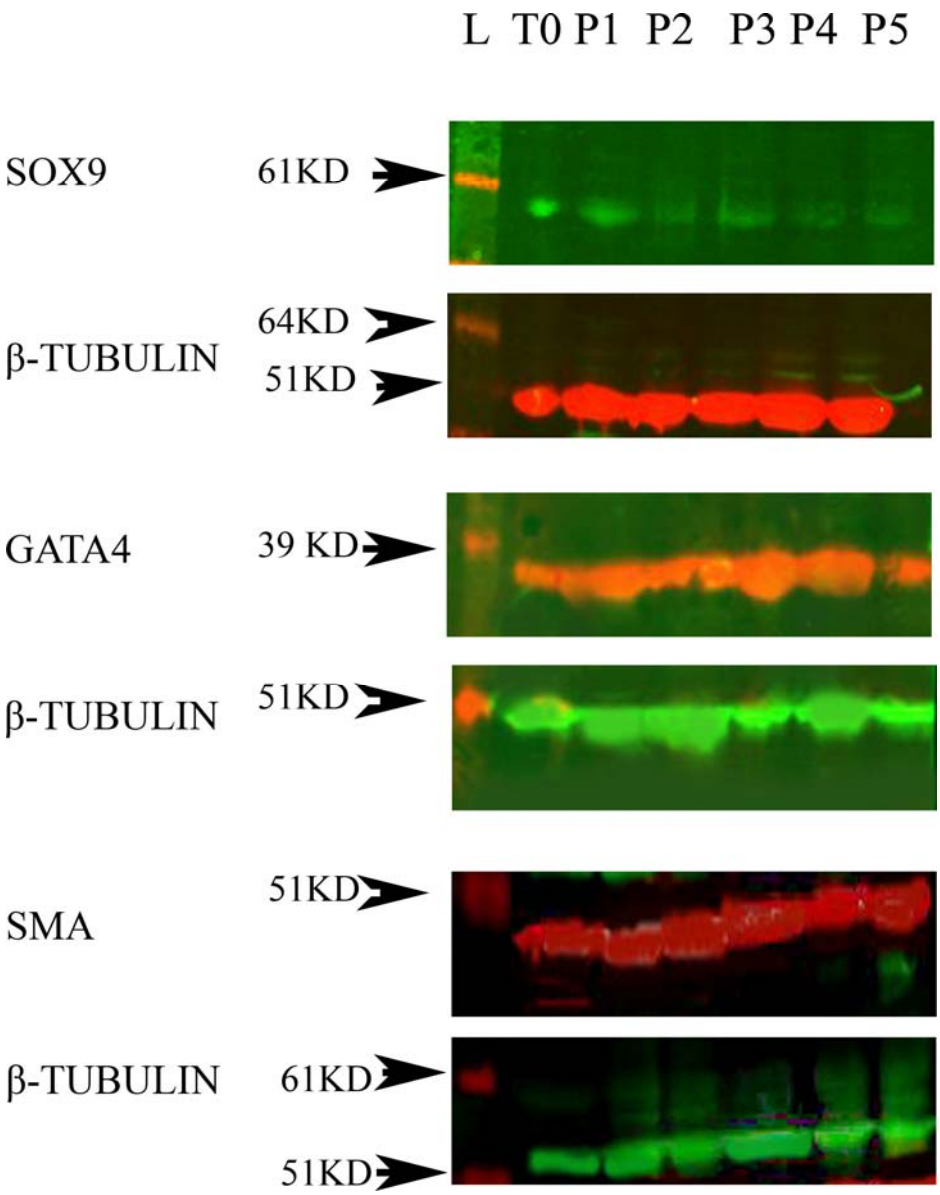


Figure 5-16 Western Blot for SOX9, GATA4 and SMA in cells from 17 week human somatic cell culture at time 0 and p1-p5.

5.4 Immunohistochemical detection of somatic markers from human fetal somatic cell cultures.

As TaqMan and Western analysis was only able to detect total levels of expression in the cell cultures, and the initial observations based on morphology suggested a mixture of cell types were present, additional analysis was carried out using

immunohistochemistry on the cell cultures at passage 1 to passage 3. SMA was immunolocalised to the cytoplasm of a subpopulation of the cells *in vitro*. It could be detected at the passages 1-3 analysed. SOX9 immunostaining revealed positive nuclear staining in a subset of the cultured somatic cells and a similar result was obtained for GATA4. SCCp450 was immunopositive in only the occasional cell at p1 and very rare cells at p2, where it was confined to the cytoplasm (Figure 5-17).

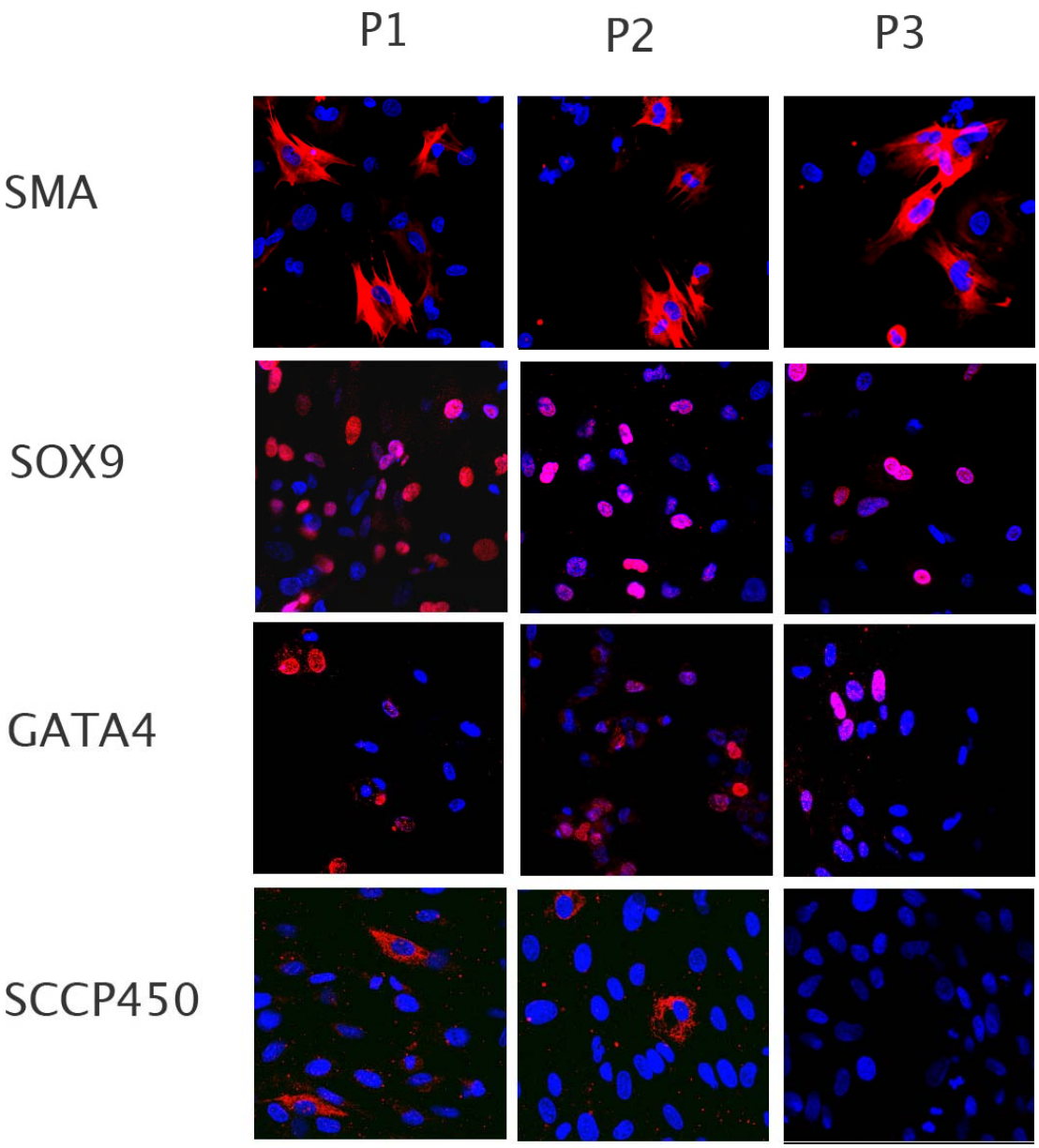


Figure 5-17 Immunostaining of cultures established from 17 week human fetal testis at p1-p3, showing expression of SOX9, GATA4, SCCp450 and SMA. SOX9, GATA4. SCCp450 and SMA are all shown in red, while Dapi is shown in blue.

Immunostaining for DES and VIM was also performed. DES immunostaining was confined to a subset of the cells (Figure 5-18 a and b). VIM immunostaining was more intense at passage 4 compared with passage 1, with a greater intensity per cell.

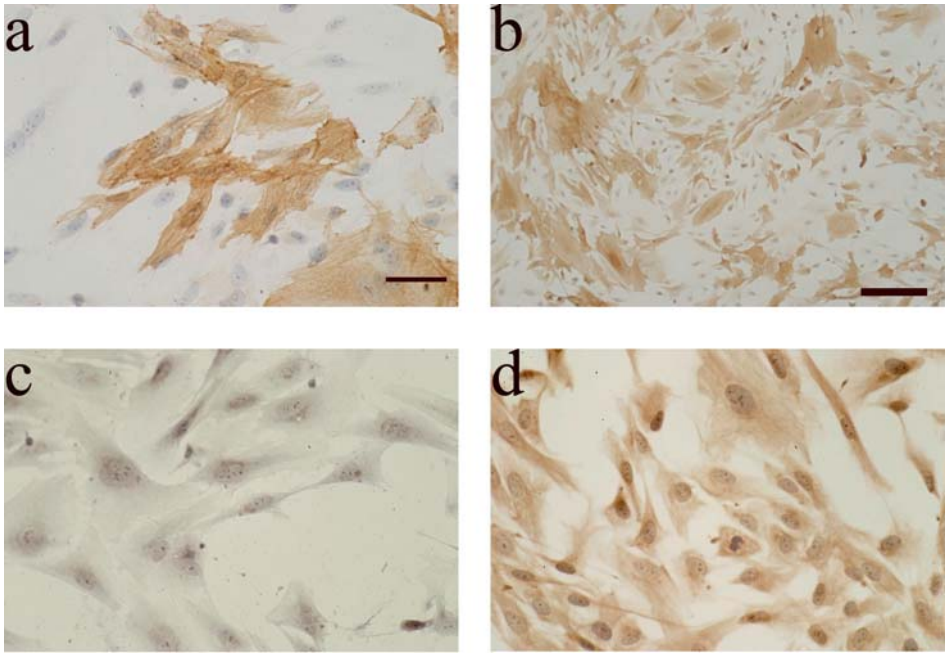


Figure 5-18 Immunostaining for DES in 18 week human fetal testis culture at a) passage 1 and b) passage 4. Immunostaining for VIM c) passage 1 and d) passage 4. Bar=100 µm for a, c and 50µm for b.

5.4.1 Activin treatment of human fetal somatic cell cultures

Since expression of both the type I and type II activin receptor mRNAs was maintained throughout the 5 passages (Figure 5-15), additional cultures were performed with or without the addition of activin in order to determine what effects, if any, activin could have on the cells and their gene expression.

The morphology or number of the cultured cells did not change in response to activin treatment (Figure 5-19).

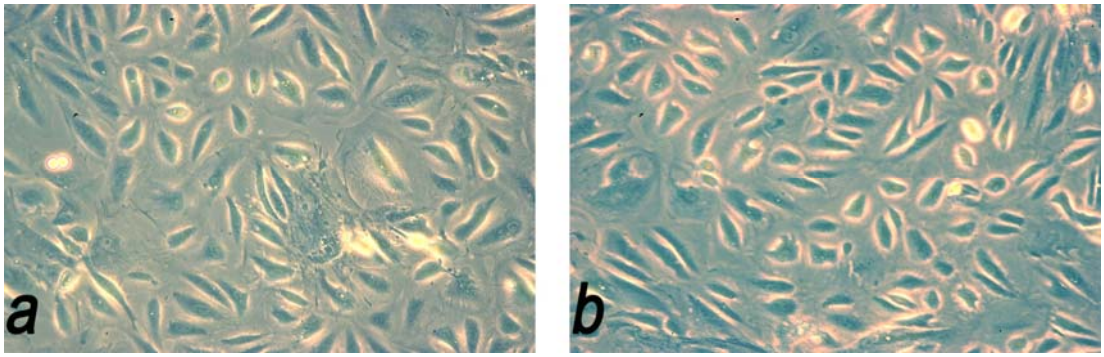


Figure 5-19 Morphology of cells after 7 days of culture for a) treatment with HBSS control b) treatment with activin

The expression of key genes required for activin signalling were maintained throughout the cultures (Figure 5-20).

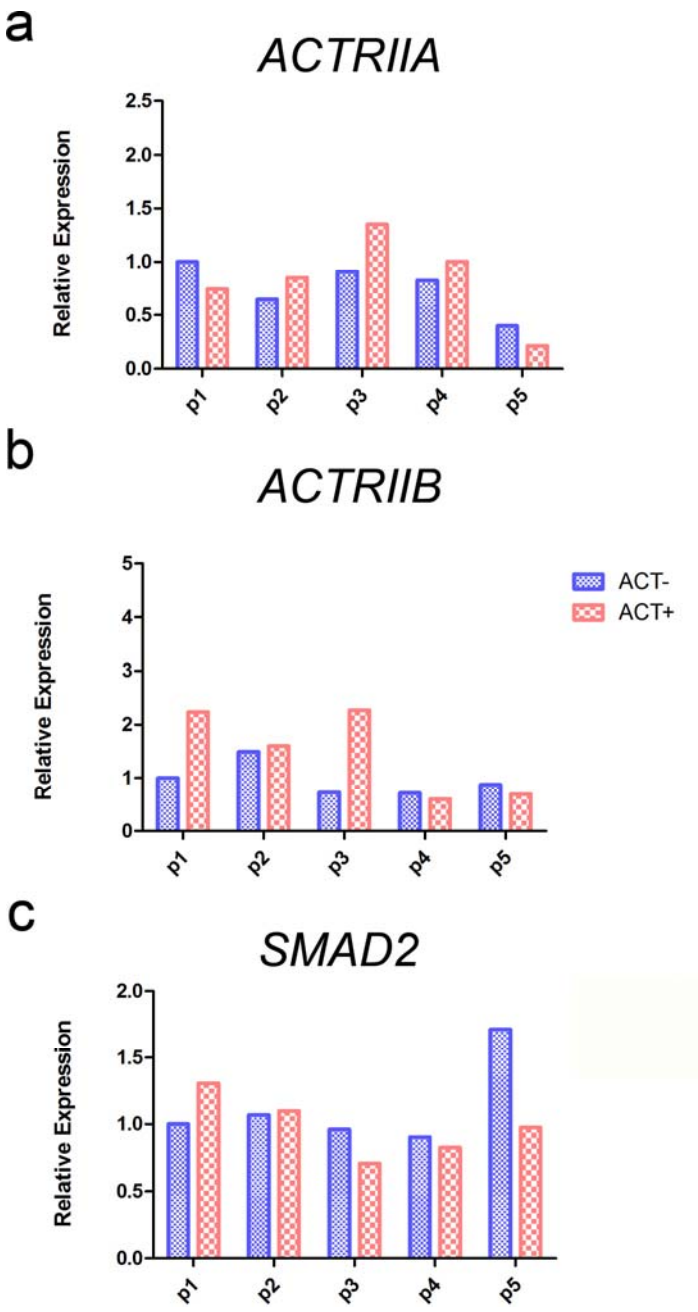


Figure 5-20 Taqman analysis of cells cultured from p1-p5 following treatment either with or without activin for a)ACTRIIA b)ACTRIIB c)SMAD2. Taqman was performed in triplicate for 2 independent cultures from fetuses obtained at 15 week and 17 weeks. Relative to dissociated 15 week testis control culture. Mean ± sem

Inclusion of activin in the culture media did not prevent loss of expression of the Sertoli cell markers *AMH* and *DHH* or the Leydig cell marker *SCCp450* during multiple passages (Figure 5-21).

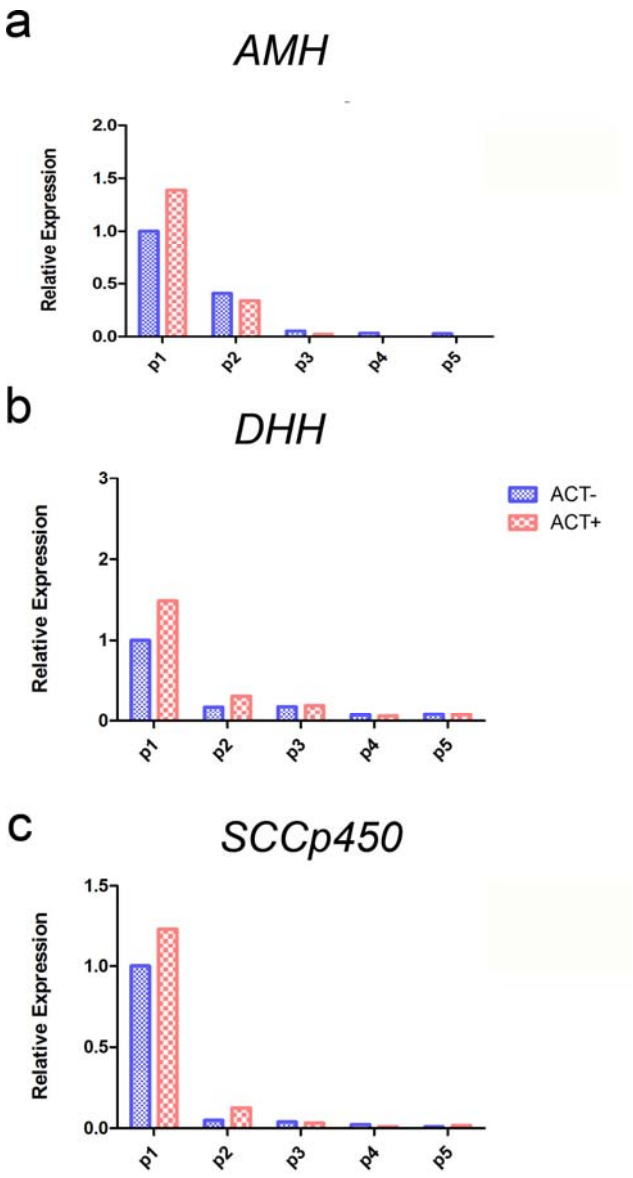


Figure 5-21 Taqman analysis for a) AMH mRNA expression b) DHH mRNA expression and c) SCCp450 mRNA expression following culture in the presence or absence of activin A, relative to 15 week control culture. Taqman was performed in triplicate for 2 independent cultures from fetuses obtained at 15 week and 17 weeks.

SOX9 and *SMA* mRNA were expressed in a similar manner to previous cultures with *SOX9* declining with increasing passage, and *SMA* increasing as the cultures progressed. But the presence of activin did not alter *SOX9* or *SMA* expression (Figure 5-22).

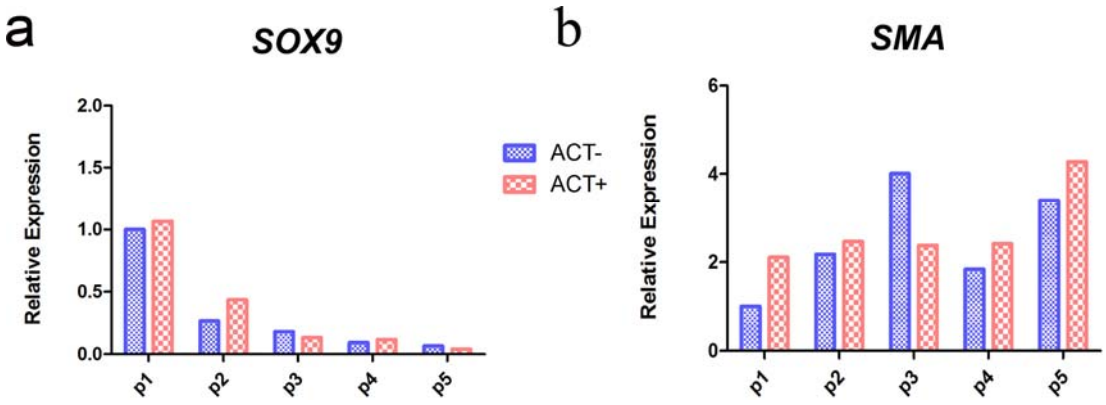


Figure 5-22 mRNA expression of a) SOX9 and b) SMA in human fetal somatic cells cultured in the presence or absence of activin A, relative to dissociated 15 week testis control culture (N=2, 15 week and 17 week). Taqman was performed in triplicate for 2 independent cultures from fetuses obtained at 15 week and 17 weeks.

5.5 The effect of trypsin/EDTA on human fetal somatic cell cultures

Since the expression of mRNAs such as *SOX9*, *SRY*, *WT1*, *SCCp450* and *3βHSD* have been shown to decrease with repeated passage in these cultures, the effect of trypsin/EDTA on the cells was analysed.

Testis were halved and dissociated as normal in the presence of Collagenase IV and DNAse1, half were then treated with trypsin/EDTA for 10 minutes, as had been performed for all previous experiments. The other half were placed in culture without the trypsin dissociation step. In testes that were dissociated in the absence of trypsin/EDTA a larger number of cells appeared to adhere and divide (Figure 5-23).

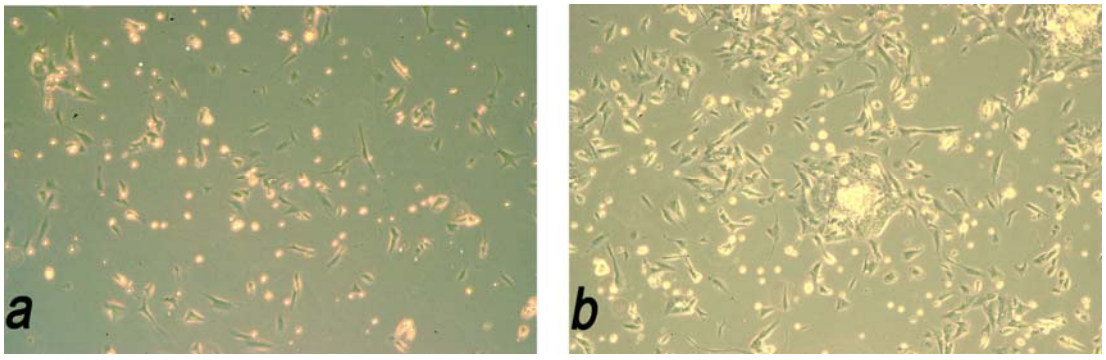


Figure 5-23 Images of cells from dissociated 14 week human fetal testis 2 days after culture when a) dissociated with trypsin/EDTA b) dissociated in the absence of trypsin/EDTA.

TaqMan analysis for the Sertoli cell genes *SOX9* and *AMH* showed increased expression after 7 days of culture when tissue was dissociated without trypsin/EDTA, as did the Leydig cell marker *SCCp450*. A student's t-test was performed but the difference between means were found to be non-significant for all three ($P>0.05$) (Figure 5-24).

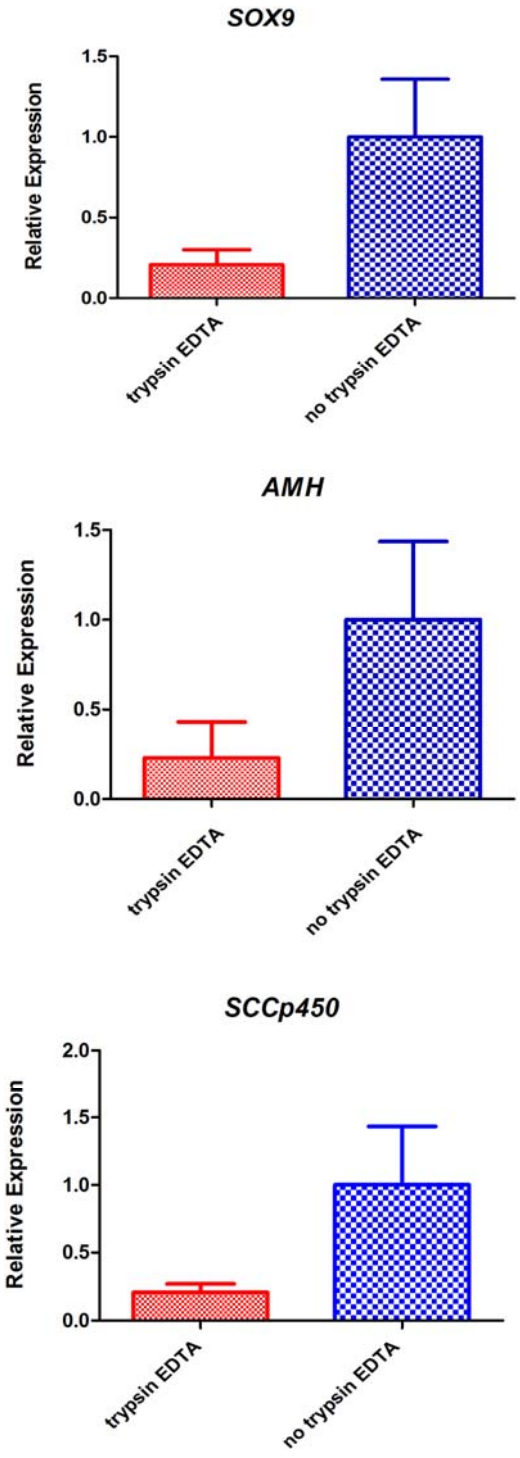


Figure 5-24 mRNA expression for SOX9, AMH and SCCp450 in human fetal somatic cell cultures after 7 days of culture following dissociation either with or without trypsin/EDTA. N=3, Relative to cultures dissociated in the absence of trypsin/EDTA. Mean ± sem

Since the use of trypsin/EDTA during the initial dissociation procedure appeared to have an adverse effect on the survival of the cells and subsequent mRNA expression, this raised concerns about continuously passaging the cells with trypsin/EDTA also. Cultures were set up where cells were passaged with dispase instead of trypsin. The effects of passaging with dispase on mRNA expression were then analysed.

Analysis of cells dissociated in the absence of trypsin/EDTA and then subsequently passaged using dispase instead of trypsin/EDTA still resulted in a loss of expression of *AMH* and *DHH* mRNAs (Figure 5-25).

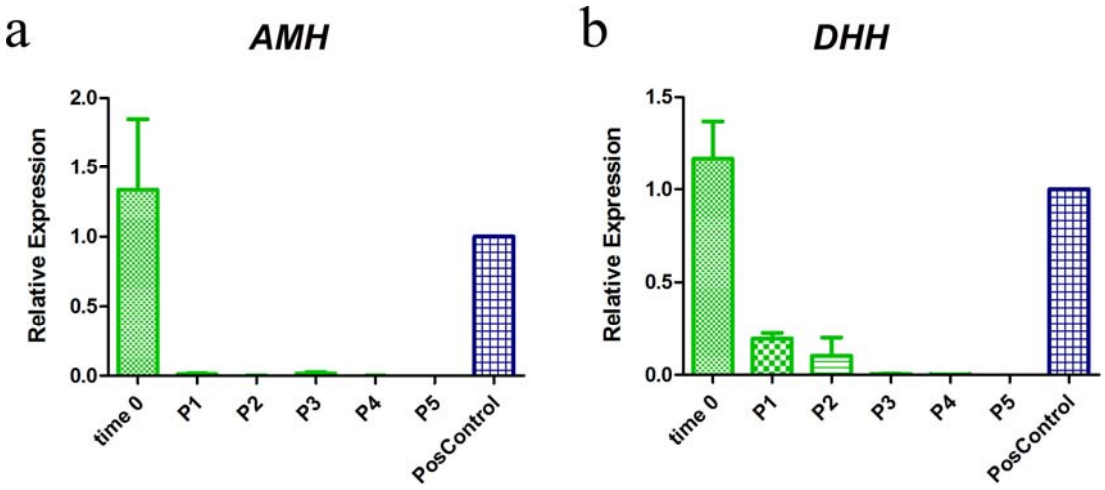


Figure 5-25 Taqman analysis of human fetal testis dissociated in the absence of trypsin and passaged using dispase a) AMH b) DHH. Taqman was performed in triplicate for 3 independent cultures from fetuses obtained at 13 weeks and 2x17 weeks. Relative to positive control. Mean ± sem

The mRNA expression of *SRY, SOX9*, and *WT1* were all maintained throughout the cultures when passaged with dispase (Figure 5-26) and did not really differ from the expression when trypsin/EDTA was used (Figure 5-10 and Figure 5-11).

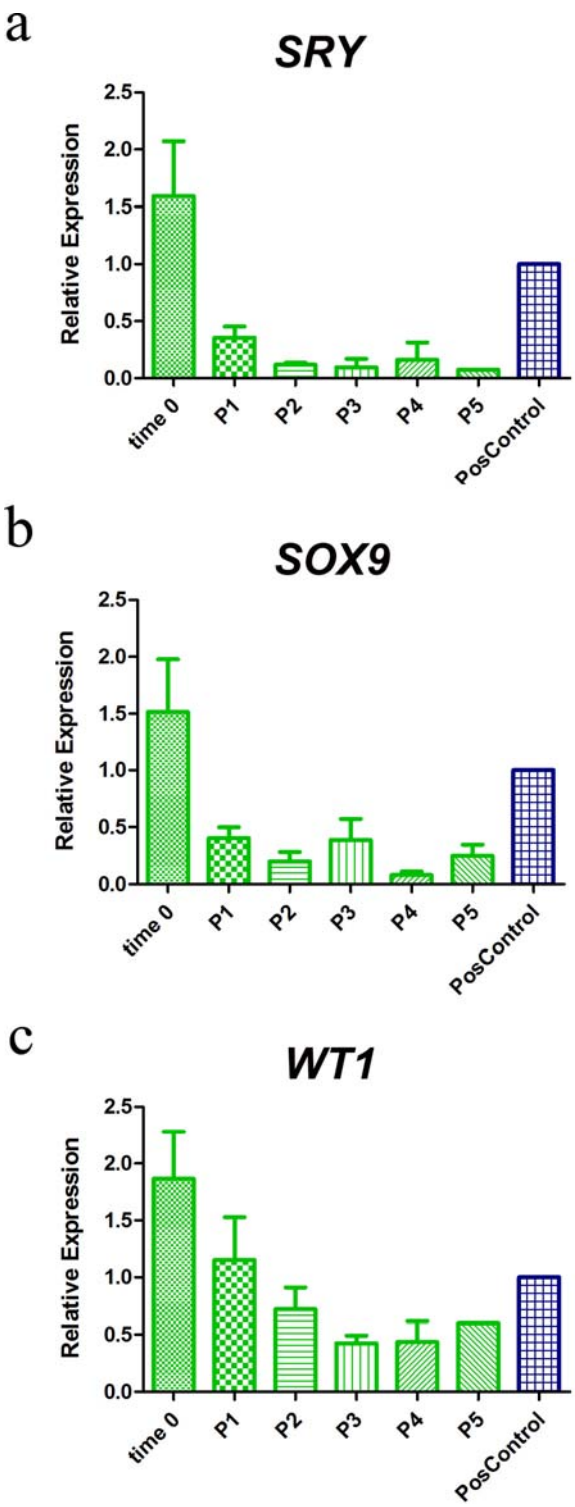


Figure 5-26 mRNA expression for a) SRY b) SOX9, and c) WT1 in human fetal testis dissociated without trypsin/EDTA and passaged using dispase, relative to positive control. Taqman was performed in triplicate for 3 independent cultures from fetuses obtained at 13 weeks and 2x17 weeks. Mean ± sem

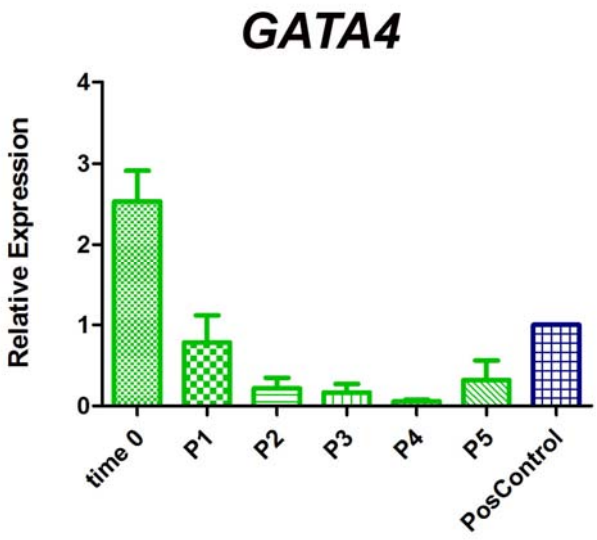


Figure 5-27 GATA4 mRNA expression in testis dissociated without trypsin/EDTA and passaged using dispase, relative to positive control. Taqman was performed in triplicate for 3 independent cultures from fetuses obtained at 13 weeks and 2x17 weeks. Mean ± sem

GATA4 mRNA levels (Figure 5-27) were maintained as before and did not appear any higher or lower than when cells were passaged with trypsin/EDTA (Figure 5-11).

Expression of *SCCp450* mRNA at p1 was comparable to that at time 0, but was not maintained thereafter even if dispase was used in place of trypsin/EDTA (Figure 5-28). In addition to total amounts of α-*SMA* mRNA still increased as cells were passaged beyond passage 2 (Figure 5-29), although levels were not as high as when cell dissociation was performed with trypsin/EDTA.

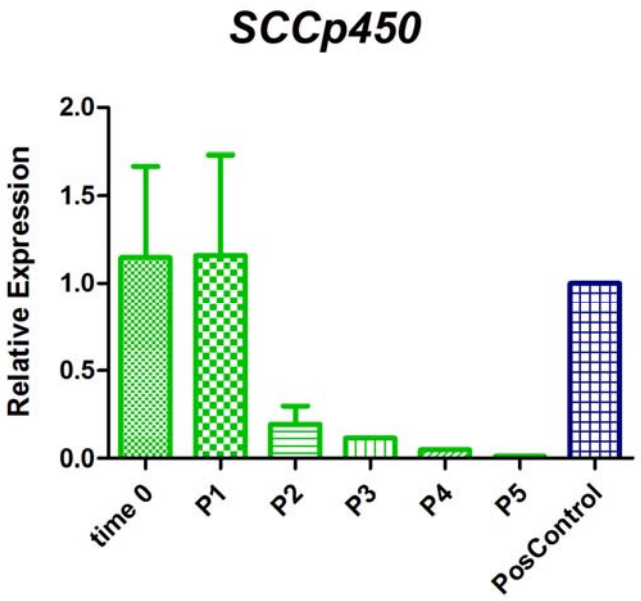


Figure 5-28 mRNA expression of SCCp450 in testis dissociated in the absence of trypsin/EDTA and passaged using dispase. Relative to positive control. Taqman was performed in triplicate for 3 independent cultures from fetuses obtained at 13 weeks and 2x17 weeks. Mean ± sem

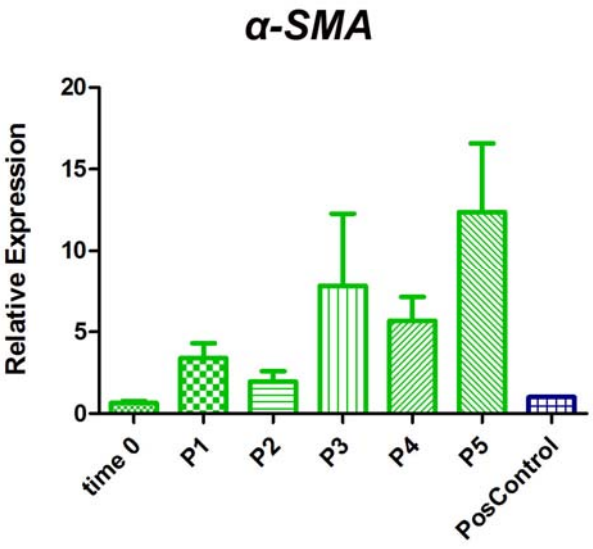


Figure 5-29 mRNA expression of α-SMA in testis dissociated in the absence of trypsin/EDTA and passaged using dispase. Relative to positive control. Taqman was performed in triplicate for 3 independent cultures from fetuses obtained at 13 weeks and 2x17 weeks. Mean ± sem

5.6 Discussion

Our ability to understand the regulation of gene expression in individual cell types within the human fetal testis would be enhanced if we were able to isolate them and culture them *in vitro*. In this study human fetal testes were dissociated into a single cell suspension, maintained *in vitro* and passaged so as to determine whether somatic cells could a) be identified and b) maintain a differentiated phenotype. Gene expression analysis was then performed on these cells for further characterisation.

5.6.1 Protein expression of somatic cell markers in the human fetal testis

Before commencing the studies with dissociated cells, immunohistochemical staining was used to identify the expression of specific cell markers for the Sertoli cell, Leydig cell and PTM cell lineages in the human fetal testis. The transcription factor SOX9 was exclusively immunolocalised to the nuclei of Sertoli cells throughout the 2nd trimester. This is in agreement with a previous report which detected *SOX9* in Sertoli cells of 1st and 2nd trimester testis (Hanley et al., 2000). AMH was localised to the cytoplasm of the Sertoli cells throughout the 2nd trimester, as has been previously demonstrated (Gaskell et al., 2004; de Santa Barbara et al., 2000). The transcription factor, WT1 was expressed in the nuclei of the Sertoli cells in the 2nd trimester, consistent with previous studies (de Santa Barbara et al., 2000; Hanley et al., 1999).

Interstitial cells in 2nd trimester testis were immunopositive for the steroidogenic enzymes 3βHSD and SCCp450. Both enzymes are required for steroid biosynthesis during which SCCp450 converts cholesterol to pregnenolone (Lieberman et al., 1984) and 3βHSD converts pregnenolone to progesterone (Simard et al., 2005). In 2004 Gaskell et al. reported the onset of 3βHSD expression in second trimester human fetal testis. Several other groups have also reported the expression of these enzymes in the Leydig cells of the human fetal testis (Murray et al., 2000; Ostrer et al., 2007; Boukari et al., 2007) and the secretion of steroids during the 2nd trimester has also been demonstrated, with testosterone levels peaking between 14 and 18

weeks gestation (Voutilainen, 1992), coincidental with the time when Leydig cells are highly proliferative (Codesal et al., 1990).

Immunohistochemical staining for GATA4 revealed that this protein was highly expressed in the Sertoli cells and PTM cells of the 2nd trimester human fetal testis, and some positive Leydig cells and interstitial fibroblasts were also observed. No morphologically identifiable germ cells were found to be GATA4 positive. GATA4 expression has previously been reported in the Sertoli cells, Leydig cells and the germ cells of the human fetal testis (Ketola et al., 2000). GATA4 and its co-factor FOG2 have been immunolocalised to human fetal testis from 7 weeks gestation, where FOG2 was cytoplasmic and GATA4 was nuclear (Ostrer et al., 2007).

SMA and DES were both detected in the cytoplasm of PTM cells and presumptive myoepithelial cells surrounding the vasculature within the interstitium of the 2nd trimester testis. Expression of SMA has recently been reported to occur in PTM and vascular cells of the human fetal testis from week 7 of development (Ostrer et al., 2007) and our study is the first demonstration of DES expression in the human fetal testis. One study examining PTM markers in the developing mouse testis, could detect neither Des or Sma within the PTM cells at e13.5 and e14.5, suggesting that in the mouse the PTM cells do not acquire a smooth muscle phenotype until later in development (Jeanes et al., 2005).

Immunoexpression of AR revealed that protein expression was restricted to the PTM cells and interstitial populations. In the adult testis both Sertoli cells and Leydig cells express the androgen receptor, but previous work on the fetal rat (Majdic et al., 1995) and fetal human (Gaskell et al., 2004; Murray et al., 2000) have not detected AR in either of these cell types. The role of PTM cells in the fetal gonad is not fully understood, but the expression of AR in these cells suggests that they may be important targets for AR and establishment of a differentiated PTM cell culture to further understand these cells was one of the goals of this study and the reason that DHT was added to the media.

With the verification of the expression of these key proteins in the 2nd trimester human fetal testis, a comprehensive analysis and characterization of the cultured human fetal somatic cells could then be performed.

5.6.2 Dissociation and culture of 2nd trimester human fetal testis leads to the differential survival of somatic cells *in vitro*

Dissociation of second trimester human fetal testis into a single cell suspension and then their subsequent culture resulted in a population of cells that could be expanded *in vitro*. Within each culture, cells displayed a mixture of sizes and morphology. Identification of cells based on morphology alone was not considered sufficient for identification of cell types and therefore gene expression analysis was performed.

TaqMan analysis for the gonocyte marker, *OCT4* and prespermatogonial marker *VASA* indicated that the adherent cell cultures did not contain germ cells. This is not entirely surprising as maintaining and expanding germ cells *in vitro* is challenging and requires a number of cytokines and the support of feeder cells (Kanatsu-Shinohara et al., 2005; Tu et al., 2007; Turnpenny et al., 2006).

Analysis of the expression of *AMH* and *DHH*, both of which are secreted by Sertoli cells *in vivo*, revealed a loss of both markers from the cultures at a very early stage. Although DHH protein expression in the human fetal testis has not been studied, TaqMan analysis revealed high levels of mRNA in whole and positive control testis. Our inability to detect *AMH* and *DHH* mRNAs in the cultures beyond passage 1 would imply that Sertoli cells did not maintain a differentiated phenotype *in vitro*, however the detection of several mRNAs and proteins for other Sertoli cell specific markers, such as *SRY* and *SOX9* was at odds with this and suggested to us that Sertoli cells were present in the cultures, but with a phenotype that appears to be less differentiated and more primitive.

AMH is expressed *in vivo* throughout the 2nd trimester (Gaskell et al., 2004). The expression of Amh has been shown to be unaffected by the absence of germ cells (Behringer et al., 1994) and there are four transcription factors, all with roles in early testis differentiation, also implicated in the control of AMH expression. These are SF1 (Giuili et al., 1997; De Santa Barbara et al., 1998; Shen et al., 1994), SOX9 (Arango et al., 1999), GATA-4 (Tremblay et al., 2001a) and WT1 (Nachtigal et al., 1998).

Sox9 has been shown to be required to bind to its specific response element within the *Amh* promoter for transcription of *Amh* (Arango et al., 1999). In the undifferentiated mouse gonad, Sox9 is present in both sexes within the cytoplasmic compartment, but in the male upon sexual differentiation, it becomes nuclear (Morais da Silva et al., 1996). This also appears to be the case in the human, where SOX9 undergoes nuclear translocation prior to AMH expression (de Santa Barbara et al., 2000). SOX9 contains a nuclear export signal sequence that regulates nucleocytoplasmic translocation of SOX9 during sex determination, and in cultured mouse XX gonads when Sox9 nuclear export was inhibited, a sex-reversal phenotype resulted, accompanied by the expression of Amh (Gasca et al., 2002). The nuclear receptor Sf1 may also have some degree of control over *Amh* transcription. Male mice homozygous for a mutant Sf1 binding site on the *Amh* promoter initiated *Amh* transcription at a significantly reduced level. Within the *Sf1* proximal promoter there are Sox9 binding sites, and functional evidence that Sox9 upregulates *Sf1* (Shen and Ingraham, 2002). Both SOX9 and SF1 have been shown to localise to somatic cells in the human fetal testis prior to both cord formation and the expression of AMH (de Santa Barbara et al., 2000).

The cooperative actions of SF1 and WT1 have been shown to be necessary for AMH upregulation (Nachtigal et al., 1998), while GATA4 has also been shown to transactivate the expression of AMH by interacting with SF1 protein (Watanabe et al., 2000) and a recent study has shown regulation of the AMH promoter by WT1 is enhanced by GATA4 (Miyamoto et al., 2008). However, in the human the functional

relevance of GATA4 and WT1 in the upregulation of AMH can be called into question given that in the human fetal gonad, both appear not to be switched on until after the upregulation of AMH (de Santa Barbara et al., 2000).

In the current study, analysis of the cultures suggested that the expression of the key genes believed to control *AMH* transcription were maintained, as was the only identified target of *DHH*, namely *SF1*. For example *SOX9, WT1, GATA4* and *SF1* were all expressed for several passages after AMH and DHH were lost. The cultured cells were also found to maintain expression of SOX9 and GATA4 protein, where importantly, expression of both was restricted to the cell nucleus. In order to evaluate whether the full transcriptional machinery necessary to maintain AMH expression exists, it will be necessary to perform further studies in order to ascertain whether expression of WT1 and SF1 proteins are also maintained within the cultured cells, and also if DAX1 and the GATA4 co-factor FOG2 are expressed.

Alternatively the Sertoli cells may be transdifferentiating into fibroblasts as a consequence of their *in vitro* environment. It is necessary to determine whether the SOX9 positive cells express SMA or other fibroblast markers. One other possibility is that the Sertoli cells may be transdifferentiating into granulosa cells. It would therefore be interesting to look at the expression of genes such as *FOXL2* (Kalfa et al, 2008) to see if genes associated with the granulosa cell lineage are switching on within the cultures.

The somatic cell cultures were also analysed for the expression of the Leydig cell markers, *SCCp450* and *3βHSD*. mRNA expression of both was downregulated at passage 1 and was not detected thereafter. The pattern of expression of both of these enzymes suggests that at the early stages of the cultures there may have been functioning Leydig cells. SCCp450 protein was detected in a small subset of the cultured cells in the initial passages. The downregulation of these Leydig cell markers suggests that the cells failed to survive or proliferate within the cultures, or that perhaps the Leydig cells survived but lost their steroidogenic capacity. As

Sertoli cells play a pivotal role in Leydig cell fate, perhaps the change in the phenotype of the Sertoli cells, and the loss of important paracrine signalling molecules such as DHH (Clark et al., 2000; Yao et al., 2002) and possibly platelet-derived growth factor α (PDGF-α) (Brennan et al., 2003) might have contributed to the loss/dedifferentiation of the Leydig cells.

5.6.3 As the cultures progress the number of fibroblasts/PTM cells increase

As the cells were passaged, the expression of α-*SMA and VIM* mRNAs continued to increase as a proportion of total mRNA, suggesting that the cells were undergoing phenotypic changes or that one population was increasing at the expense of another. An increase in the expression of these markers at passage 1 and 2 can be expected relative to whole fetal testes extracts because the cultures lack germ cells or significant numbers of Leydig cells. On tissue sections α-SMA was localised to the PTM cells, and cells surrounding the blood vessels within the fetal testis. Furthermore immunohistochemical staining of α-SMA in the human fetal testis revealed no expression of α-SMA within the interstitial fibroblasts. However previous reports have shown that fibroblasts from a number of tissues convert to a myofibroblast phenotype as a consequence of their *in vitro* environment (Dugina et al., 1998). DES is another marker of myofibroblast cells (Bellin et al., 1999), while VIM is an intermediate filament protein that is characteristically expressed in fibroblast cells (Hay, 1995). The increasing expression of fibroblast markers relative to the total cell mRNA is consistent with the observed alterations in the morphology of the cells. For example, with increasing passage the number of long flat cells increased. Also with increasing numbers of passage the time until confluency was reached reduced, suggesting increasing numbers of rapidly dividing cells were overtaking the cultures. Within primary tissue cultures, elimination of fibroblasts has proven difficult with even small numbers of fibroblasts rapidly overgrowing most other cell types, especially when cultured in the presence of serum (Singer et al., 1989). There are a number of methods for eliminating fibroblasts from culture, such as selective trypsinization (Schumann et al, 1988), culture in serum-free media or

isolation with the monoclonal antibody AS02 which binds specifically with human fibroblasts (Saalbach et al., 1997) but these can prove challenging and further studies will be needed to see if they can be used for human fetal testicular cultures (Halaban and Alfano, 1984; Saalbach et al., 1997).

Throughout the five passages analysed, *AR* mRNA was present within the cultures. Despite the fact that the expression of fibroblast/PTM markers increased, *AR* expression decreased. This is surprising since these are the cells *in vivo* that express AR. This implies some loss in the differentiated phenotype in the PTM/fibroblast cells, suggesting that PTM cells may be transforming into cells with a myofibroblast cell phenotype.

5.6.4 Activin treatment had no effect on human fetal somatic cells *in vitro*

Previous studies have demonstrated the expression of activin subunits and receptors (Majdic et al., 1997; Anderson et al., 2002), suggesting a possible role for the activins in the control of human fetal testis development. In these studies, somatic cell cultures were reported to express the type II activin receptors *ACTRIIB* and *ACTRIIA*, the Type I receptors *ALK2* and *ALK4* and the downstream signalling molecule *SMAD2*. However in the current study treatment of dissociated human fetal testis with activin A at a concentration of 10 ng/ml had no effect on the morphology or survival of the cells or any of the Sertoli cell or Leydig cell mRNAs analysed. The expression of the activin receptor proteins within the cultures was not analysed and phosphorylation of SMAD2 was not studied to establish whether the cells were responsive to activin, and further experiments are therefore required to ensure that the signalling machinery remains intact. Previous reports suggest that in some models, cells responding to activin significantly upregulate *SMAD2* mRNA (Coutts et al., 2007a), but no upregulation following activin treatment was found in this study. No specific function of activin has been demonstrated in the human fetal testis, and it is necessary to elucidate the functions of activin receptor signalling further in the fetal testis to understand whether or not it has a role.

5.6.5 Trypsin has an adverse effect on the survival of human fetal testicular somatic cells *in vitro*

When human fetal testes were dissociated without trypsin, an increased number of cells survived, and larger primary colonies formed. The initial dissociation of the tissue with collagenase IV and DNase 1 and without trypsin/EDTA lead to a non-significant increase in the expression of some Sertoli cell and Leydig cell specific mRNAs, when compared with tissue dissociated with trypsin/EDTA, suggesting that trypsin/EDTA may be detrimental to the *in vitro* survival of Sertoli cells and Leydig cells. Trypsin is a pancreatic serine protease with specificity for peptide bonds involving the carboxyl group of basic amino acids: arginine and lysine (Brown and Wold, 1973). Dispase is a neutral protease that hydrolyses N-terminal peptide bonds of nonpolar amino acid residues (Kitano and Okada, 1983). Studies suggest that dispase is less harsh than trypsin (Kohnert and Hehmke, 1986; Gragnani et al., 2008). However continuous passage with dispase resulted in no real improvements to gene expression, suggesting that the method of dissociation was not a key factor in the observed changes in gene expression.

5.6.6 Conclusions

A method for the *in vitro* culture of human fetal somatic cells has been established, there are currently no reports in the literature of the culture of somatic cells from the human fetal testis. The novel findings of this thesis have demonstrated that the long-term culture of these cells results in the downregulation in expression of mRNAs specific for Sertoli cells and Leydig cells, suggesting that these cells either did not survive or thatalterations occurred to their phenotype. But this system may be able to be used as a model system for studying Sertoli cell development. If studies are to be carried out on cell signalling or cell-cell interactions using this culture system, they must be done within a short-time period, i.e within the initial 1-2 passages.

6 Expression of germ cell associated genes in mouse embryonic stem cells

6.1 Introduction

The early stages of germ cell specification and development have been extensively studied *in vivo* in the mouse (Chapter 1, section 1.3.1.1). Another model system for studying germ cell commitment and differentiation may be through the use of embryonic stem (ES) cells. ES cells are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst (Evans and Kaufman, 1981). ES cells undergo self-renewal *in vitro*, and are capable of differentiating into cell types from all three primary germ layers (ectoderm, endoderm and mesoderm) thus with the potential to differentiate into all tissue types.

Several studies have now shown that ES cells can give rise to cells that appear to belong to the germ cell lineage (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004). Different methodologies have been used to generate ES cell-derived germ cells (refer to Chapter 1, section 1.5.1). For example, Hubner et al in 2003 reported the formation of follicle-like structures which contained oocytes following the differentiation of ES cells on a monolayer, while in the same year, Toyooka et al reported the generation of ES cell-derived haploid spermatids, after the differentiation of ES cells in suspension culture, and the subsequent aggregation of putative germ cells with mouse gonadal tissue. Geijsen et al (2004) utilised an alternative approach with Oct4-GFP reporter ES cells, which were allowed to form embryoid bodies for around three weeks in the presence of retinoic acid (RA). These embryoid body-derived germ cells expressed early germ cell markers and approximately 0.01% resembled post-meiotic male germ cells. Haploid spermatids were isolated by flow cytometry and when injected intracytoplasmically into mouse oocytes, blastocysts expressing GFP were reported to develop. More recently, the production of live mice from ES cell -derived germ cells has been reported (Nayernia et al., 2006), although as yet no other groups have repeated this.

Although ES cells are often assumed to most closely resemble pluripotent epiblast cells derived directly from the inner cell mass (ICM) of the blastocyst, there are several genes which are expressed by undifferentiated ES cells and are also expressed highly in the developing germ cells.

For example, early germ cells express key pluripotency-associated proteins such as Oct4, the homeodomain-containing protein Nanog and high mobility group (HMG) domain-containing family member, Sox2 (Chapter 1, section 1.3.1.5). All three genes are highly expressed in undifferentiated ES cells and are required to maintain pluripotency (Nichols et al., 1998; Chambers et al., 2003; Avilion et al., 2003). In the embryo, initially Oct4 is expressed throughout the epiblast but it becomes restricted to the PGC cluster by e7.5 (Scholer et al., 1990; Yeom et al., 1996). PGCs continue to express Oct4 as they are proliferating and migrating to the genital ridge. Oct4 may have an important role in the survival of PGCs, as its germ cell specific ablation results in germ cell loss by apoptosis (Kehler et al., 2004). *Nanog* is also expressed in mouse PGCs, but although it is expressed in the epiblast, it cannot be detected in PGCs of e6.5-e7.5 embryos. Instead it is upregulated in PGCs between e7.75-e12.5, and is then later downregulated in female germ cells at e13.5-e14.5, and male germ cells 1-2 days later (Yamaguchi et al., 2005). *Sox2* which also expressed in the inner cell mass of the blastocyst and neural progenitor cells (Foshay and Gallicano, 2008), is expressed in the mouse germ cell lineage e6.75 (Yabuta et al., 2006) and lost at e14.5 (Western et al., 2005; Perrett et al., 2008).

Other genes expressed by the early germ cell lineage include, B-lymphocyte-induced maturation protein-1 (*Blimp1*/*Prdm1)* (Vincent et al., 2005), *Fragilis (Ifitm3)* and *Stella/PGC7* (*Dppa3*) (Sato et al., 2002; Saitou et al., 2002) (Chapter 1, section 1.3.1.4). In addition its' roles in a number of other tissues, *Blimp1* has been described as a master regulator of germ cell fate and downregulates genes associated with the somatic cell lineage such as the *Hox* genes (Yabuta et al., 2006) (Chapter 1, section 1.3.1.6). Once within the genital ridge, germ cells also express the RNA

binding protein Dazl (Seligman and Page, 1998) and the RNA helicase, mouse vasa homologue (Mvh/Ddx4) (Toyooka et al., 2000).

The expression of both S*tella (Dppa3)* and *Fragilis (Ifitm3)* have been demonstrated in undifferentiated ES cells (Geijsen et al., 2004; Pain et al., 2005). Some groups have reported the absence of *Mvh* in undifferentiated ES cell lines (Toyooka et al., 2003), while others have found it to be present in the undifferentiated state (Kerkis et al., 2007) and the RNA-binding protein Dazl has also been reportedly expressed in undifferentiated mouse ES cells (Geijsen et al., 2004; Kerkis et al., 2007) .

The tyrosine kinase receptor, *Kit* is also expressed in both germ cells and ES cells (Palmqvist et al., 2005), despite being absent form the ICM (Horie et al., 1991; Matsui et al., 1990). The Kit receptor is activated by the Kitl (or stem cell factor) (Chapter 1, section 1.3.1.7). In the mouse in the absence of both Kit and Kitl, germ cells die by apoptosis (Mintz and Russell, 1957; Chabot et al., 1988; Nocka et al., 1989; Matsui et al., 1990; Pesce et al., 1993). Additionally Kitl has been shown to be required for the successful *in vitro* culture of PGCs (De Felici and Dolci, 1991; Dolci et al., 1993; Matsui et al., 1991). The Kit/Kitl signalling pathway acts to promote the survival of many progenitor cells; in addition to its role in germ cells, it plays an important role in maintaining haematopoietic, neuronal and melanocytes stem cells (Kent et al., 2008; Wehrle-Haller, 2003).

Within ES cells, Kit signalling appears to be important as they undergo differentiation. Bashamboo et al (2006) have generated mouse ES cell lines carrying null alleles of *Kit* (W^{lacZ}), these mutant cells underwent apoptosis when they were differentiated upon withdrawal of LIF, suggesting that Kit/Kitl signalling is required as the cells differentiate. Interestingly, preliminary data from gene profiling suggested that the expression of several germ cell genes was reduced within the *Kit* null ES cell line (Bashamboo and Forrester, unpublished).

6.1.1 Aims of Chapter

This study firstly aimed to investigate germ cell gene expression in mouse ES cells in order to understand further the overlap in gene expression between germ cells and embryonic stem cells. The expression of germ cell genes was analysed in the three wild-type ES cell lines (CGR8, E14 and EFC1) in order to determine whether they were expressed and if so what degree of variability there was between the cell lines. RNA extracted from mouse testes at e13.5 or adult, were included to allow a direct comparison with levels of expression *in vivo*. The second aim of this study was to determine whether Kit signalling had an influence on germ cell gene expression in mouse ES cells. Germ cell gene expression was analysed in ES cell lines that had undergone immunomagnetic bead sorting, using an antibody directed against Kit, in order to obtain *Kit* 'low' and *Kit* 'high' populations of cells. Additionally germ cell gene expression was also analysed in mutant ES cell lines that were heterozygous and null for the *Kit* receptor.

6.2 Material and Methods

6.2.1 ES cell culture

6.2.1.1 ES cell lines

E14 (Hooper et al., 1987), CGR8 (Mountford et al., 1994), and EFC1 (Nichols et al., 1990) embryonic stemES cell lines were cultured in feeder- free culture conditions in the presence of LIF (10ng/ml) (Sigma). Additional cultures were prepared using the *Kit* null cell line (Kit $^{w\text{-}lacz}$/Kit $^{w\text{-}lacz}$) and the heterozygous cell line (Kit $^{w\text{-}lacz}$/Kit $^{+}$), both of which were derived from the parental cell line E14 (Bashamboo et al., 2006).

6.2.1.2 Cell thawing

Cells were thawed rapidly by placing them in a water bath at 37^{o}C. Media was then added and cells were then centrifuged at 1200 rpm for 3 minutes. The supernatant was removed and the cells were resuspended in media (section 2.7); cells were

placed in a 25cm flask, precoated with 0.01% gelatin. Media was changed after a few hours.

6.2.1.3 Passaging ES cells

Following a wash with PBS, confluent cells were subjected to 2 ml of trypsinization (TVP) (section 2.7) and incubated for 3-5 minutes. The cells were removed and placed in a centrifuge tube with 8mls of media, and centrifuged at 1000 rpm for 5 minutes. Thereafter, cells were resuspended in 10 ml of media, supplemented with 10 ng/ml of LIF and a cell count was performed; 1-1.5 $x10^6$ cells were added to a T25 flask, which had been pre-coated with 0.1% gelatin. Cells were normally confluent after 2-3 days of culture.

6.2.2 Embryoid body formation

Confluent undifferentiated ES cells were harvested and then placed in suspension culture at a density of 1-2 million cells/ml in a 35mm bacterial grade petri dish in with GMEM containing 10% FCS without LIF.

6.2.3 Immunomagnetic bead sorting

E14 and EFC1 cells were grown to confluency and harvested for immunomagnetic bead sorting. Cells were washed in PBS and centrifuged at 800 rpm for 5 minutes. This was repeated twice. Cells were resuspended in PBS with 0.1% BSA to obtain 10 $x10^6$ cells/ml. 10% sheep serum was added and incubated for 10 minutes. A sample of unsorted cells was taken, and lysed for RNA extraction. Sheep anti-rabbit Dynabeads were vortexed and the required volume was transferred into an eppendorf. The tube was placed in the magnet for 2 minutes, and then the supernatant was removed. The tube was removed from the magnet and an excess volume of wash buffer was added (PBS+0.1% BSA) and the beads were resuspended. This wash step was repeated twice.

The cells were then incubated with the primary antibody (Rabbit anti-Kit, Santa Cruz); this was added in excess to block all antigen binding sites on the cell surface. The cells were incubated in 2μg of primary antibody per 10^7 cells for 30 minutes at

4°C on a rocking platform. After incubation, the cells were centrifuged at 1000 rpm for 10 minutes, the supernatant with the unbound immunoglobulin was discarded, and the cells were resuspended in PBS containing 0.1% BSA, this step was repeated twice.

The cells were then incubated with 1x 10^7 Dynabeads per ml of sample to give approximately 4 beads per target cell. The washed beads were added to the antibody covered cells and incubated for 30 minutes at 4°C on a rocking platform. After the incubation period, the cell/Dynabead suspension was placed in a Dynal MPC-L magnet (Dynal A.S, Oslo. Norway) and allowed to stand for 2 minutes. The supernatant was removed, centrifuged at 1000 rpm for 10 minutes and the cells lysed for RNA extraction. Cells bound to the beads were also lysed immediately for RNA extraction.

6.2.4 RNA extraction from tissues and cells

RNA extraction was performed as outlined in section 2.4

6.2.5 Preparation of cDNA using random hexamers

cDNA was prepared as detailed in section 2.5

6.2.6 TaqMan® analysis

TaqMan® analysis was performed using the Roche Universal Probe Library. Details of the TaqMan® reaction and analysis are detailed in section 2.5. Primers used and corresponding probe number are listed in Table 6.1.

Table 6-1 Summary of primers and probes used for TaqMan reaction

Gene Name	5' sequence	3' sequence	Probe
Oct4	gaggctacaggacacctttc	gtgccaaagtggggacct	6
Nanog	agcctccagcagatgcaa	ggttttgaaaccaggtcttaacc	25
Sox2	tccaaaaactaatcacaacaatcg	gaagtgcaattgggatgaaaa	63
Blimp1	tgcggagaggctccacta	gttgctttccgtttgtgtga	80
Fragilis	tggtctggtccctgttcaa	ccatcttccgatccctagact	27
Stella	gatgcacaacgatccagattt	tggaaattagaacgtacatactccaa	73
Dazl	gctgatattttgcccaatgaa	atgcttcggtccacagactt	78
Mvh	aagcagagggttttccaagc	gcctgatgcttctgaatcg	83
Kit	gatctgctcgtcgtcctgtt	cttgcagatggctgagacg	15
Kitl	agcgctgcctttccttatg	tccttggttttgacaagaggat	68
Stra8	accgtggtggccttaaaga	atcatcactgggttggttgc	80
Hoxa1	aaaagaaaccctcccaaaaca	tgaagtggaactccttctccag	100

For the TaqMan® analysis, data was always made relative to a selected E14, bound, wild-type or undifferentiated value. Statistical analysis was performed using either a Student's t-test or a one-way ANOVA, followed by a Bonferroni's multiple comparison test.

6.2.7 Western Blotting

Western blotting was performed on protein extracted from cultured ES cells. Cells were lysed in RIPA buffer (section 2.6.1) and protein was quantified using a Biorad protein assay (section 2.6.2). Details of Western blotting are outlined in section 2.6. Information regarding the primary antibodies used is listed in Table 6-2.

Table 6-2 Details of primary antibodies used for Western Blotting

Antigen	Host Species	Source	Dilution
Mvh	Rabbit	AbCam	1:500
β-Tubulin	Mouse	Sigma	1:1000

6.3 Results

6.3.1 mRNA analysis of germ cell markers in undifferentiated wild-type ES cell lines

6.3.1.1 Expression of Kit and Kitl

Analysis of the mRNA expression of the *Kit* receptor and *Kitl* was performed in ES cell lines and adult mouse testis. *Kit* mRNA was expressed in all cell lines examined, although at a lower level than adult mouse testis. *Kitl* mRNAs were detectable in all the cell lines examined (Figure 6-1), although at lower levels than in the adult mouse testis.

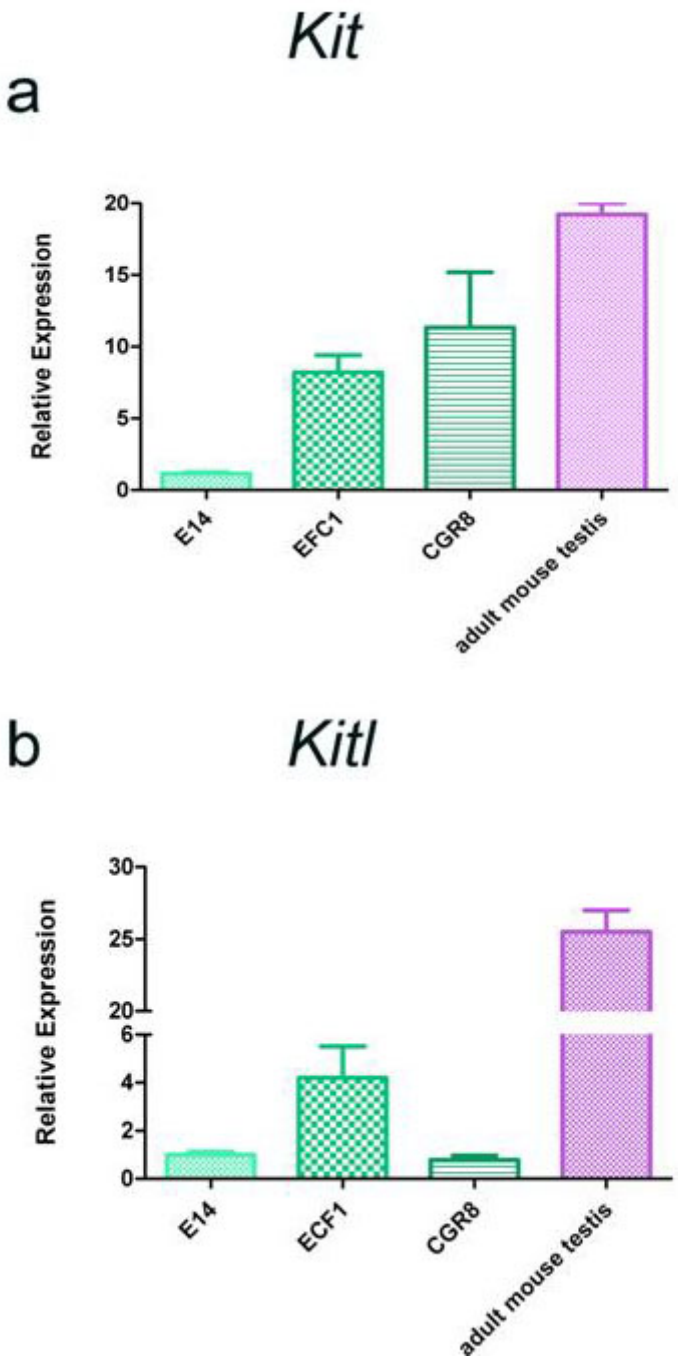


Figure 6-1 mRNA analysis of a) Kit and b) Kitl in undifferentiated E14 cells, EFC1 cells, CGR8 cell lines and positive control adults mouse testis, relative to E14 cells (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.1.2 Expression of pluripotency markers

TaqMan analysis was performed to evaluate the levels of expression of pluripotency genes in the three ES cell lines. Expression of *Oct4, Nanog* and *Sox2* mRNAs were higher in undifferentiated E14, EFC1 and CGR8 cell lines than in total embryonic mouse testes (Figure 6-2 a, b and c respectively). The amount of *Oct4*, *Nanog* and *Sox2* mRNAs were not significantly different between the three cell lines.

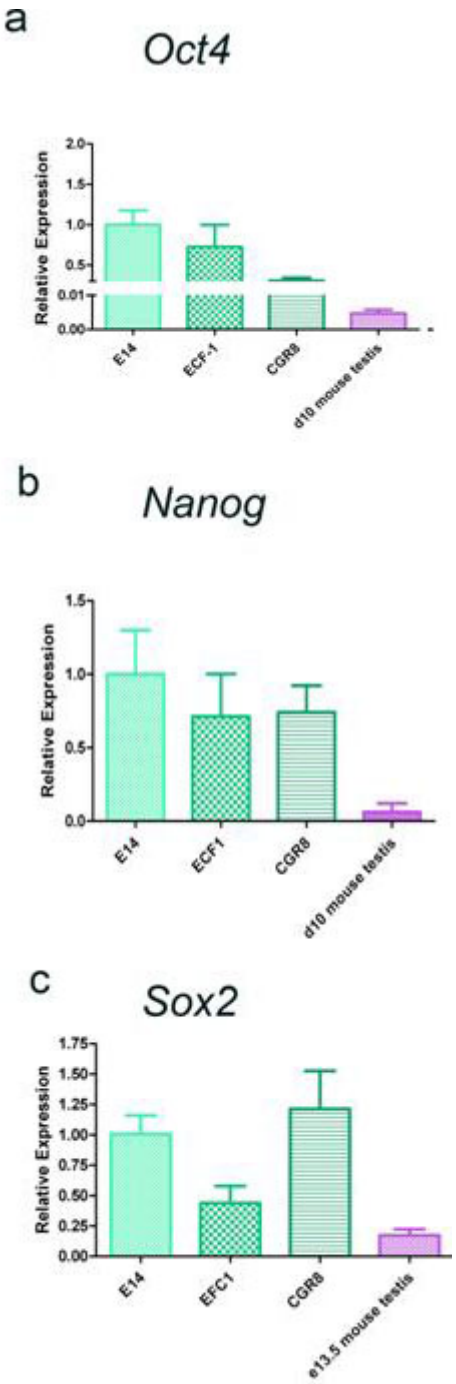


Figure 6-2 mRNA analysis for a) Oct4 b) Nanog and c) Sox2 in undifferentiated E14 cells, EFC1 cells and CGR8 cell lines and e13.5 mouse testis positive control, relative to a selected E14 (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.1.3 Expression of early germ cell markers

The expression of early germ cell marker in ES cells was analysed, as was the expression in e13.5 mouse testis. The amount of *Blimp1* mRNA was indistinguishable between the three ES cell lines examined (Figure 6-3a). Likewise, *Fragilis* mRNA was expressed in all three ES cell lines, although expression was significantly different between EFC1 and CGR8 cells ($P<0.05$). *Stella* mRNA was expressed in E14, EFC1 and CGR8 cells, although in the EFC1 cell line, expression of *Stella* was significantly higher than E14 and CGR8 cells ($P<0.05$).

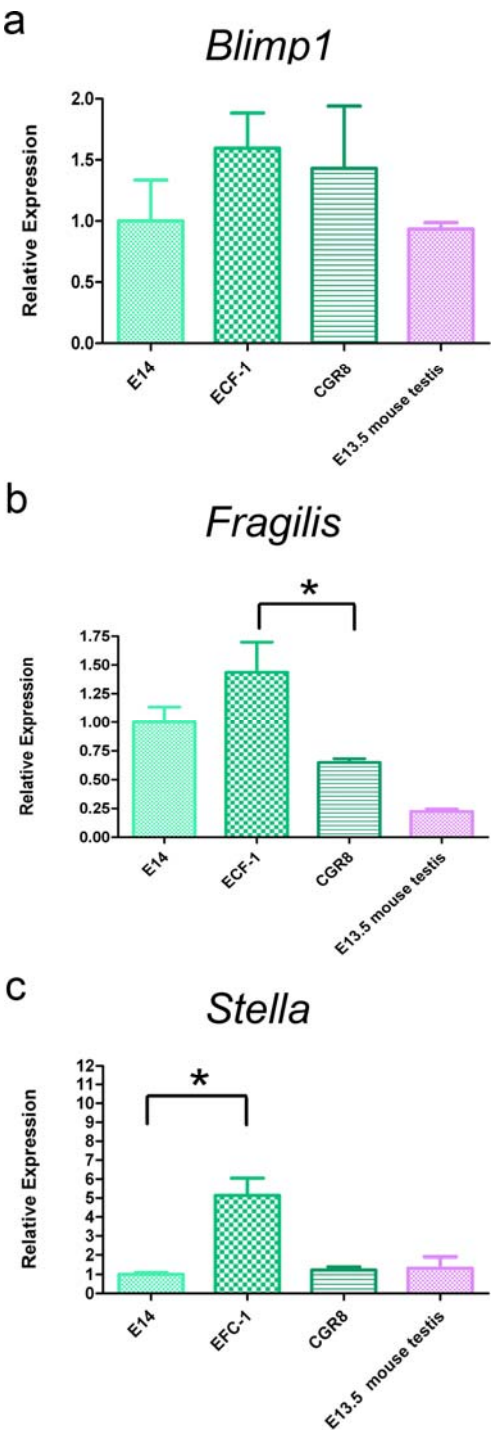


Figure 6-3 mRNA analysis of a) Blimp1 b) Fragilis and c) Stella in undifferentiated E14 cells, EFC1 cells and CGR8 cell lines and e13.5 mouse testis, relative to E14 cells (N=4). For Fragilis, EFC1 was significantly different from E14 cells (P<0.05). For Stella EFC1 was significantly different from E14 cells (P<0.05). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.1.4 Expression of late germ cell markers

The expression of later germ cell markers was analysed in the wild-type cell lines and adult mouse testis. *Dazl* and *Mvh* mRNAs were detectable in the three ES cell lines analysed at lower levels than in adult mouse testis (Figure 6-4). Both of these later germ cell markers were therefore expressed at far higher levels in the adult mouse testis compared with the undifferentiated cell lines.

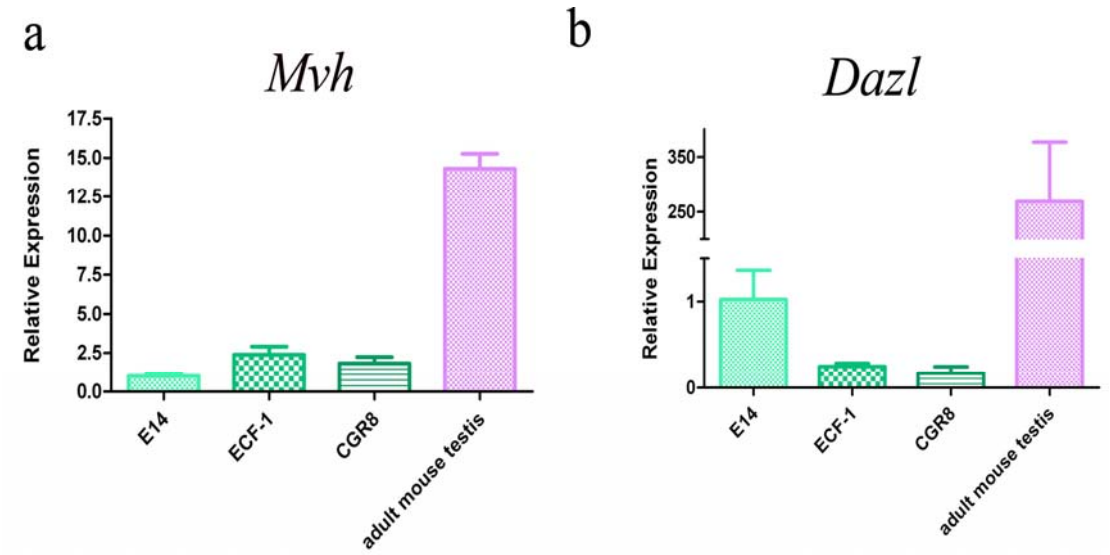


Figure 6-4 mRNA analysis of a) Mvh and b) Dazl in undifferentiated E14 cells, EFC1 cells, CGR8 cell lines and positive control adult mouse testis, relative to E14 cells (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

The amount of *Stra8* mRNA expressed by all three the cell lines although levels were lower than in the adult mouse testis (Figure 6-5). *Stra8* mRNA concentrations were slightly higher in CGR8 cells than in E14 and EFC1.

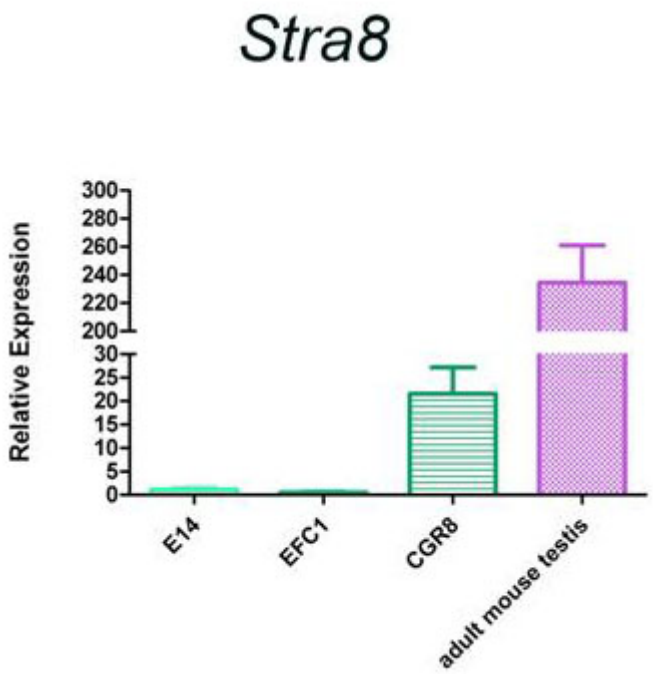


Figure 6-5 mRNA analysis of Stra8 in undifferentiated E14, EFC1 and CGR8 cell lines and adult mouse testis, relative to E14 cells (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.1.5 Expression of Hoxa1

Hoxa1 mRNA was expressed in all three ES cell lines at fairly similar levels, although at lower levels than in adult mouse testis (Figure 6-6).

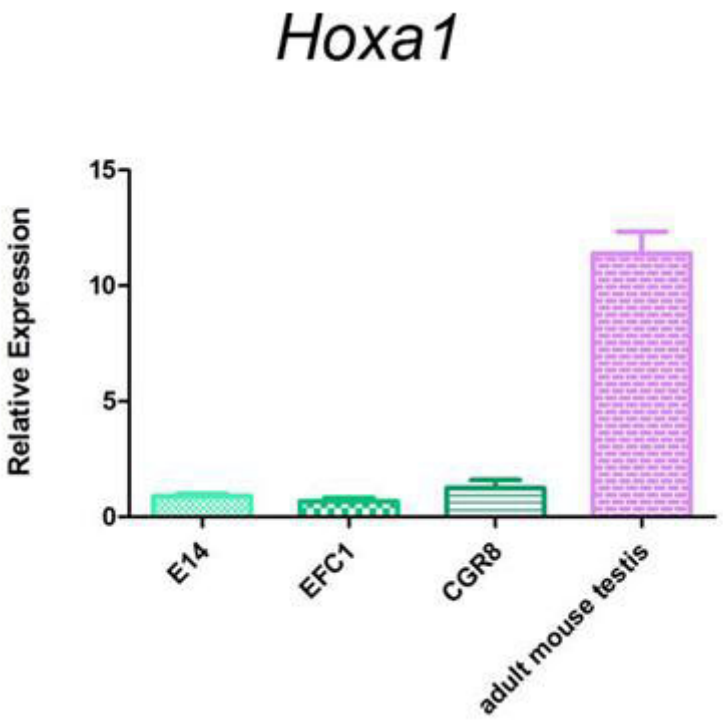


Figure 6-6 mRNA analysis of Hoxa1 in E14, EFC1 and CGR8 cell lines and adult mouse testis, relative to E14 cells (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.2 mRNA analysis of germ cell markers in day 5 embryoid bodies

E14 ES cells were allowed to grow in suspension culture, in the absence of LIF, to promote the formation of aggregates or embryoid bodies. After 5 days of culture, mRNA expression analysis was performed in order to measure germ cell and pluripotency markers. The expression of *Oct4* and *Nanog* decreased significantly ($P<0.01$ for *Oct4* and $P<0.05$ for *Nanog*) in embryoid bodies compared with the undifferentiated cells. There was also some decrease in expression of *Blimp1* and *Fragilis,* while *Stella* mRNA levels were significantly reduced ($P<0.05$). The expression of both *Kit* and *Kitl* decreased with differentiation. (Figure 6-7a), and there was also a slight decrease in the expression of *Stra8 mRNA*, (Figure 6-7a). Notably expression of *Mvh* and *Dazl* mRNAs were higher in day 5 embryoid bodies compared with undifferentiated ES cells. There was a slight decrease in the expression of *Stra8 mRNA*, while *Hoxa1* mRNA expression appeared to increase

slightly with differentiation, although these changes did not reach significance (Figure 6-7b).

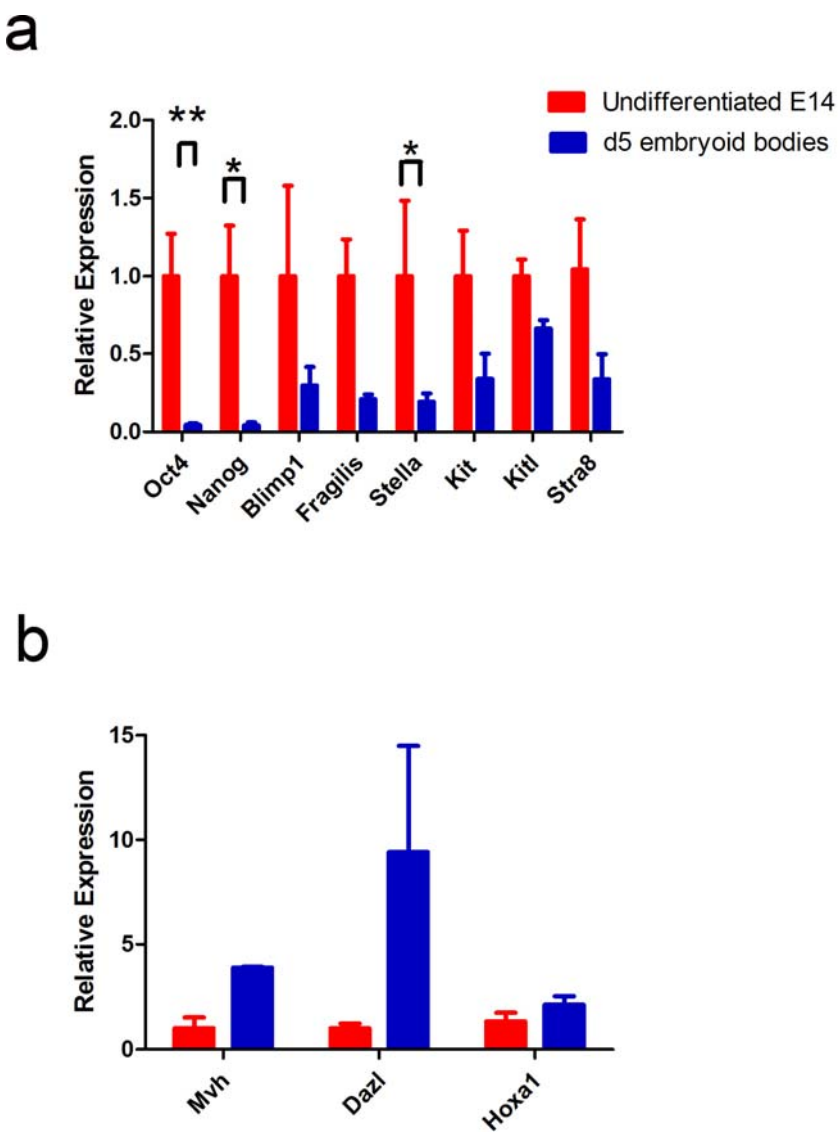


Figure 6-7 Relative changes in gene expression following ES cell differentiation (day 5 embryoid bodies). Relative to a selected undifferentiated ES cell values (N=4). a) mRNAs that decrease upon differentiation b) mRNAs which increase with differentiation. Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.3 Analysis of protein expression in E14, EFC1 and CGR8 cell lines

Western analysis for total protein concentrations revealed that *Mvh* (76kD), was present in E14, EFC1 and CGR8 cell lines; overall levels concentrations appeared similar for all 3 cell lines (Figure 6-8).

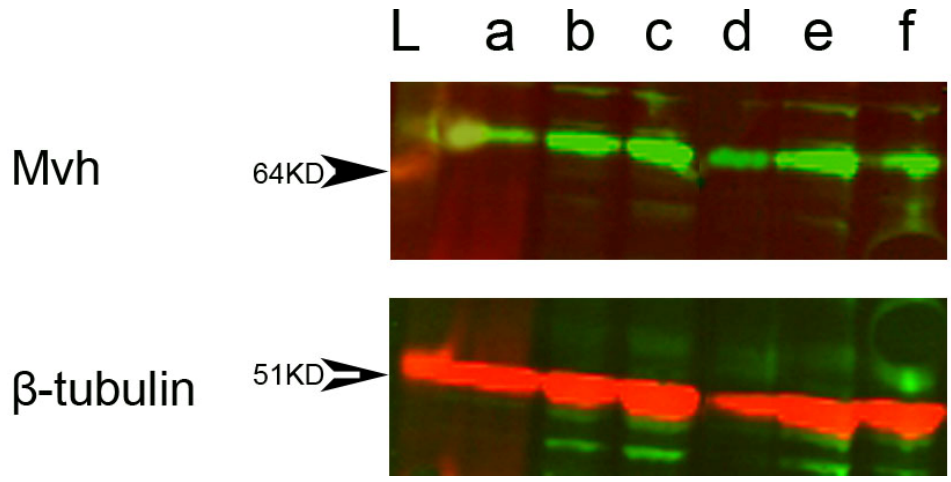


Figure 6-8 Western analysis of total Mvh protein in ES cell lines. β-tubulin is loading control. Lane L is the ladder, lane a and b are E14 cells, c and d, are EFC1 cells and e and f are CGR8 cells

6.3.4 mRNA analysis of germ cell markers in ES cells sorted into Kit high and Kit low populations using immunomagnetic beads

To separate ES cells into two populations of cells, one which expressed high levels of Kit and one which expressed low levels of Kit, immunomagnetic bead sorting was performed using an antibody directed against Kit

6.3.4.1 Expression of Kit and Kitl

Immunomagnetic bead sorting resulted in a significantly higher expression of *Kit* mRNA in the bound fraction of the sorted cells for both E14 ($P<0.05$) and EFC1 cells ($P<0.05$) (Figure 6-9), revealing that both of these cell lines did not express *Kit* uniformally in all cells.

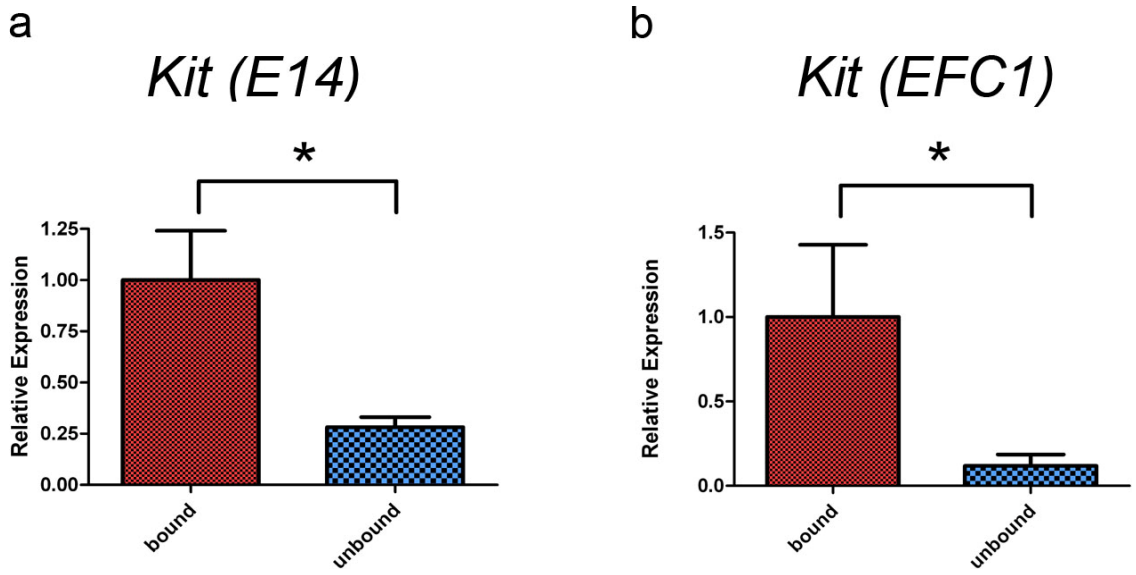


Figure 6-9 mRNA analysis for Kit expression in sorted and unsorted EFC1 and E14 cells, relative to bound cells (N=4). Taqman was performed in triplicate for 4 independent cell sorts. Mean ± sem

6.3.4.2 Expression of pluripotency genes

Expression of *Oct4*, *Nanog* and *Sox2* mRNA did not vary between the bound and unbound fraction of cells and this was consistent for both E14 and EFC1 cell lines (Figure 6-10) and suggests that there are no differences in the expression of these genes would be consistent with the lack of any difference in these cell markers in the E14 *Kit* 'high' and *Kit* 'low' populations. In general the expression of pluripotency genes does not appear to be different in *Kit* 'high' or *Kit* 'low' populations and thus appeared not to be related to *Kit* expression.

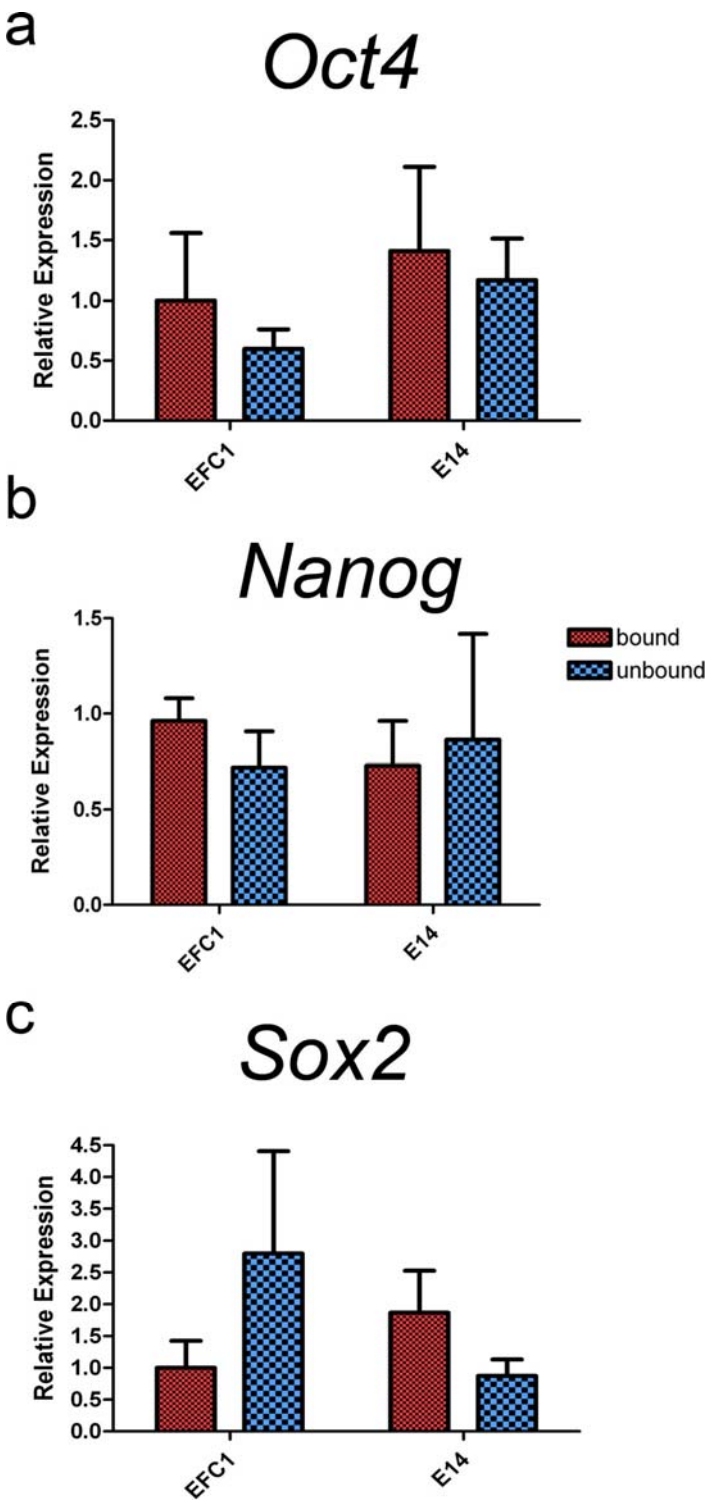


Figure 6-10 mRNA expression for a) Oct4 b) Nanog and c) Sox2 in bound (Kit 'high') population and unbound (Kit 'low') population for EFC1 and E14 cell lines, relative to bound population (N=4). Taqman was performed in triplicate for 4 independent cell sorts. Mean ± sem

6.3.4.3 Expression of early germ cell markers

Expression of *Blimp1* mRNA was significantly lower in the unbound (*kit* 'low') population of cells for both EFC1 ($P<0.01$) and E14 populations ($P<0.01$) (Figure 6-11a). Expression of *Fragilis* mRNA was tended to be lower in the unbound population (kit 'low') EFC1 cells, but this was non-significant (Figure 6-11b). Expression of *Stella* mRNA levels were non-significantly different in the unbound

(*Kit* 'low') population for both EFC1 and E14 cells (Figure 6-11c).

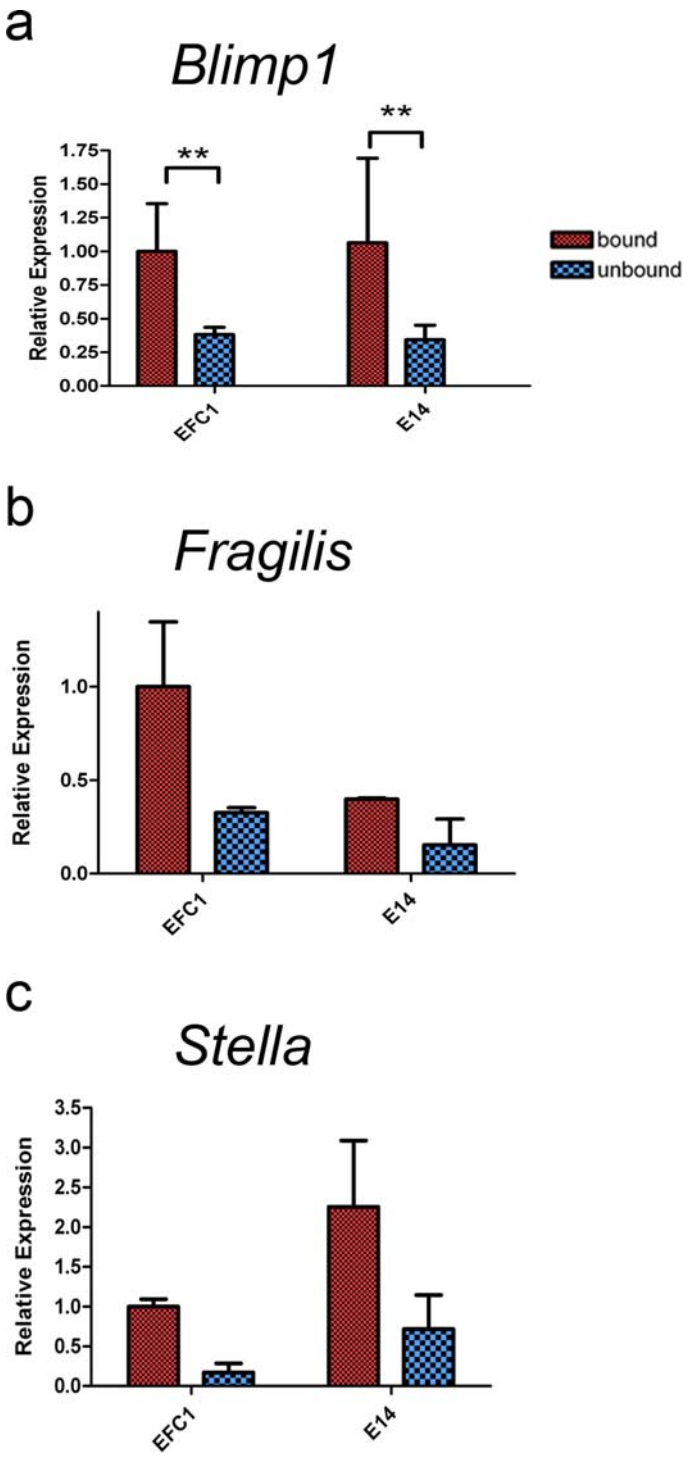


Figure 6-11 mRNA expression for a) Blimp1 b) Stella and c) Fragilis in Kit positive and negative population of EFC1 and E14 cell lines. Taqman was performed in triplicate for 4 independent cell sorts. Mean ± sem

6.3.4.4 Expression of late germ cell markers

Mvh and *Dazl* mRNA levels did not vary between the bound and unbound fraction (Figure 6-12). So within the Kit 'low' and Kit 'high' populations the expression of late germ cell markers does not vary in undifferentiated ES cells.

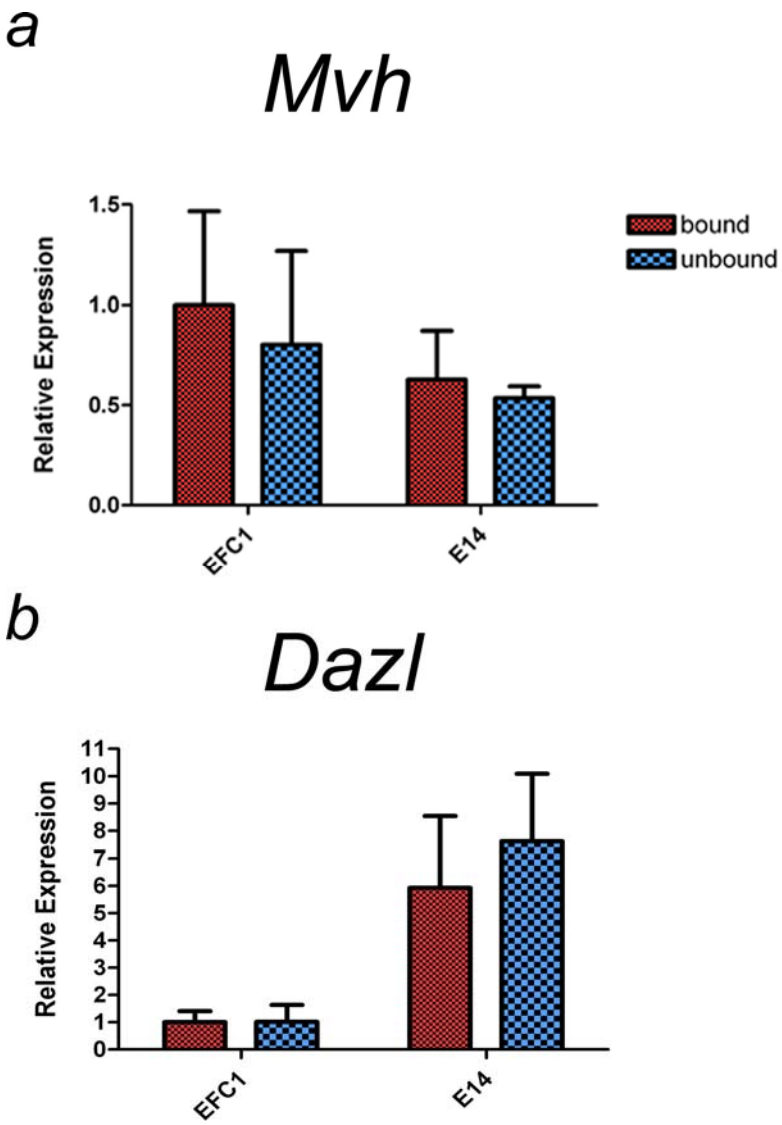


Figure 6-12 mRNA expression for Dazl and Mvh in Kit positive and negative population for EFC1 and E14 cell lines. Taqman was performed in triplicate for 4 independent cell sorts. Mean ± sem

6.3.4.5 Expression of *Hoxa1*

Hoxa1 mRNA levels were slightly higher in the unbound fraction of cells although there were no significant differences between the bound and unbound populations of EFC1 and E14 cells (Figure 6-13).

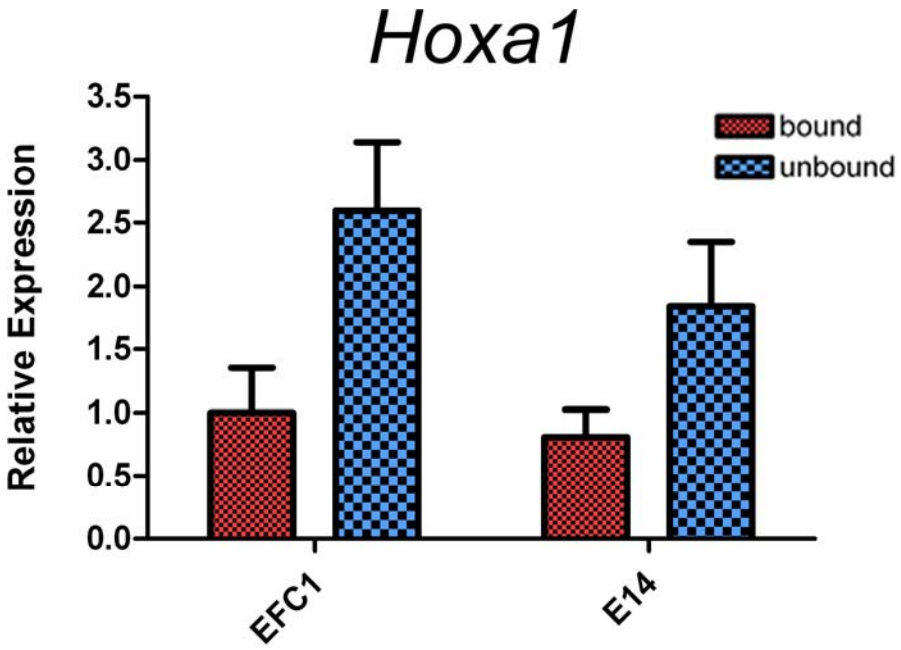


Figure 6-13 mRNA expression of Hoxa1 mRNA in Kit positive and negative populations of EFC1 and E14 cell lines. Taqman was performed in triplicate for 4 independent cell sorts. Mean ± sem

6.3.5 mRNA analysis of germ cell markers in undifferentiated wild-type, heterozygous (Kit $^{w\text{-}lacz}$/Kit $^{+}$) and null cells (Kit $^{w\text{-}lacz}$/Kit $^{w\text{-}lacz}$)

Gene profiling analysis suggested that the *Kit* null cell expressed reduced concentrations of germ cell mRNAs compared with parental cells (Bashamboo and Forrester, Unpublished). The heatmap in Figure 6-14 shows the expression of selected genes in the two null cells generated by Bashamboo et al, FKBLacZ and LacZLacZ. For both *Kit* null cell lines in the presence of LIF (plus), there was a decrease in the early germ cell genes, *Blimp1, Stella* and *Fragilis.* Gene expression in other lineages was also analysed. Genes associated with the cardiomyocyte

lineage such as the NK2 transcription factor related, locus 5 (*Nkx2.5)*, the myocyte enhancer factor 2C, *Mef2c*; the neuronal markers nestin (*Nes*) and the neuronal cell adhesion molecule *L1 Cam*; the smooth muscle specific alpha actin (*Acta2*); and the adipocyte associated gene, peroxisome proliferator-activated receptor*γ* (*Pparγ)* showed little differences in the wild-type compared with the null cells.

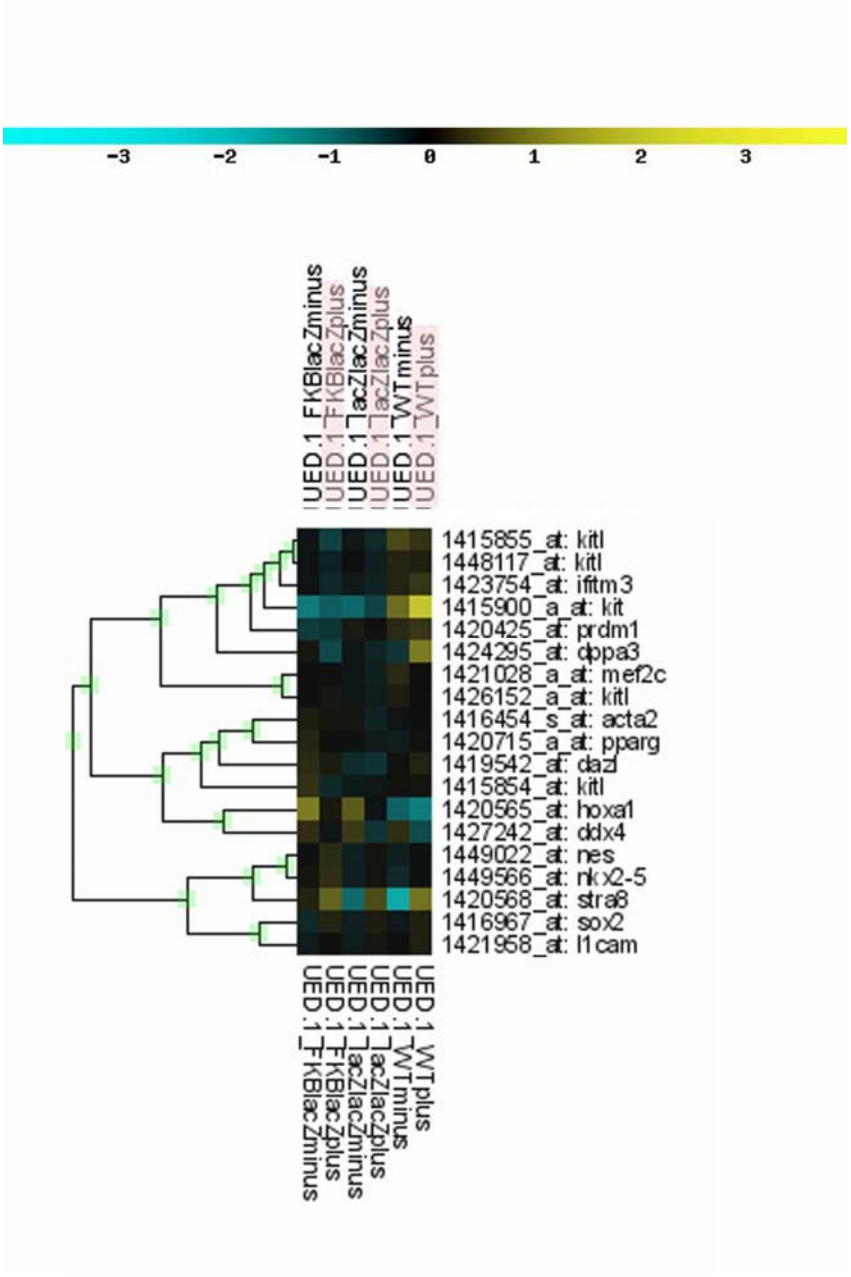


Figure 6-14 Heatmap generated from array performed by Bashamboo and Forrester. Obtained from Functional Genomics in Embryonic Stem cells (Fungenes). http://www.bioinf.ebc/ee/fungenes. Pink bands indicate the conditions where the cells have been cultured in the presence of LIF.

Quantitative RTPCR was carried out to compare mRNA expression E14 wild-type, the heterozygous (Kit $^{w\text{-}lacz}$/Kit $^{+}$) and the null cells (Kit $^{w\text{-}lacz}$/Kit $^{w\text{-}lacz}$) in order to confirm or expand upon these findings.

6.3.5.1 Expression of Kit and Kitl

Consistent with the genotype of the cells, *Kit* mRNA was undetectable in the null cells and was reduced in the heterozygous cells compared with the wild-type cells (Figure 6-15a). Notably the reduced expression of the receptors had no impact on expression of *Kitl* mRNA and these were comparable between wild-type, heterozygous and null cells (Figure 6-15b), thus the null cells maintain expression of *Kitl* in the absence of *Kit.*

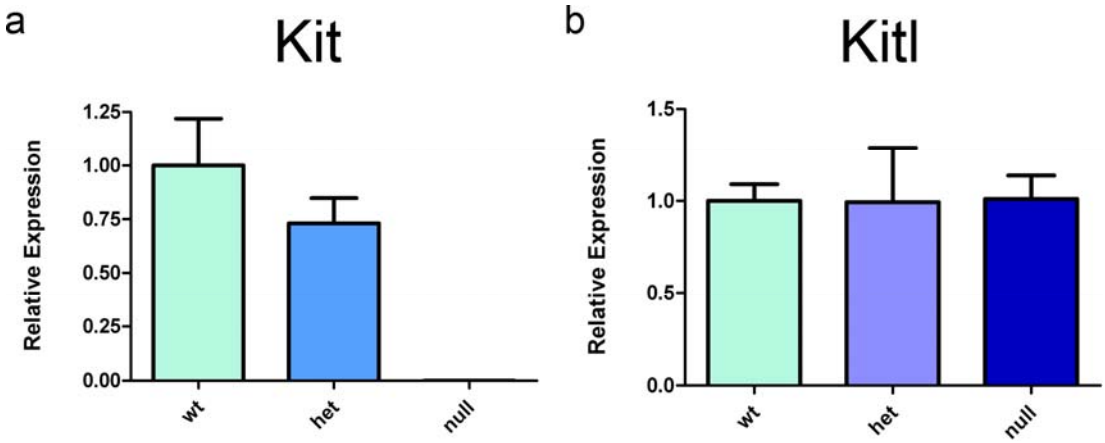


Figure 6-15 mRNA analysis for Kit and Kitl in wild-type, heterozygous and null cells, relative to wt (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.5.2 Expression of pluripotency genes

Oct4, *Nanog* and *Sox2* mRNAs were all detectable in wild-type, heterozygous and null undifferentiated E14 ES cells, with expression of all three mRNAs at relatively similar levels (Figure 6-16), suggesting that Kit signalling is not required for expression of these genes.

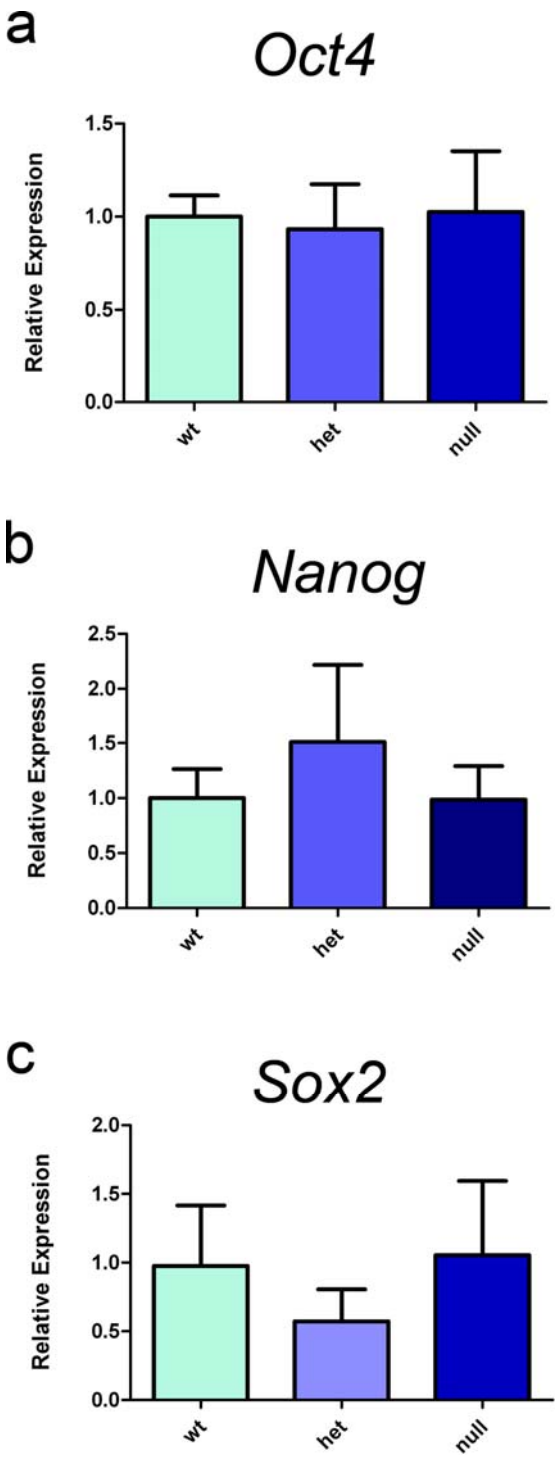


Figure 6-16 mRNA analysis of a) Oct4 b) Nanog and c) Sox2 in wild-type (wt), heterozygous (het) and null cells, relative to selected wt value (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.5.3 Expression of early germ cell markers

Expression of *Blimp1* mRNA was significantly lower in heterozygous ($P<0.01$) and null cells ($P<0.001$) than wild-type cells ($P<0.05$) (Figure 6-17a). Expression of *Fragilis* was significantly lower in the null cells compared with wild-type cells ($P=0.05$) (Figure 6-17b). Expression of *Stella* was significantly reduced relative to wild-type cells ($P<0.05$), with heterozygous cells also displaying slightly lower levels of mRNA expression compared with wild-type cells (Figure 6-17c). These confirm the array data, where early germ cell gene expression does seem to vary between the Kit cell lines. The *Kit* null cells express significantly lower levels of all of these germ cell genes.

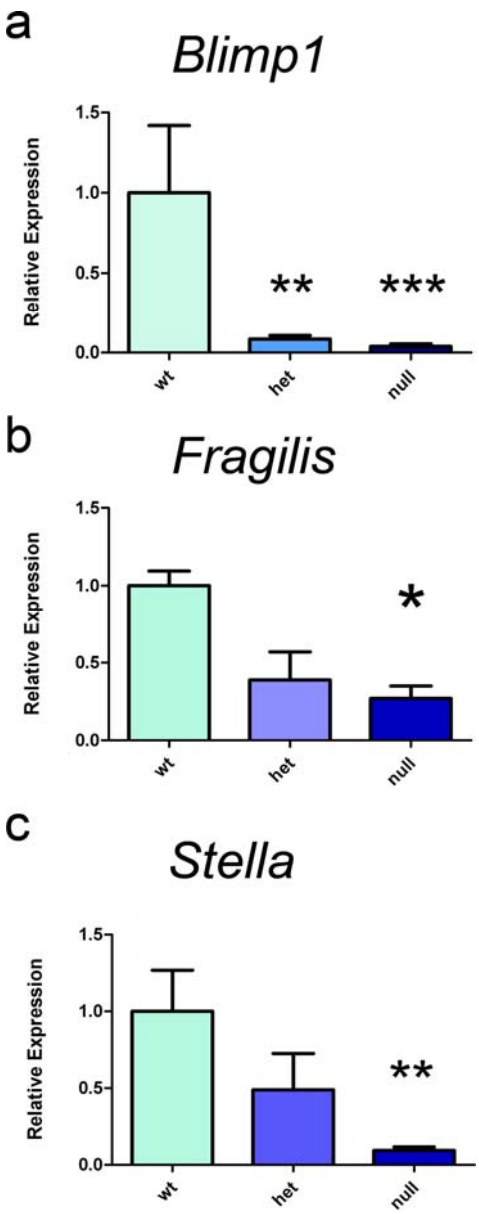


Figure 6-17 mRNA analysis of a) Blimp1 b) Fragilis and c) Stella in wild-type (wt), heterozygous (het) and null cells, relative to wt (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.5.4 Expression of late germ cell markers

The expression of both *Dazl* and *Mvh* mRNAs were similar in the null, wild-type and the heterozygous cells (Figure 6-18 a and b). Therefore there are no significant differences in late germ cell marker gene expression between the wild-type, heterozygous and null cells.

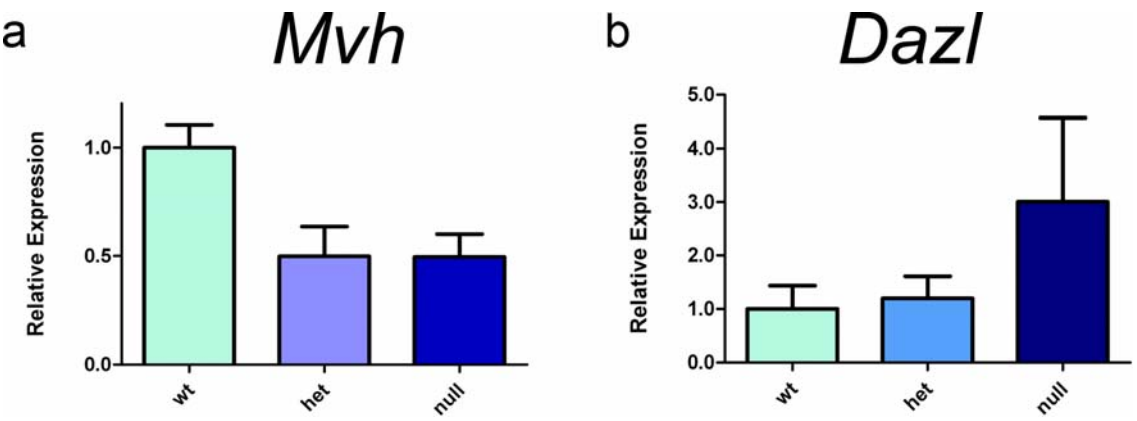


Figure 6-18 mRNA analysis for a) Mvh and b) Dazl in wild type wt, heterozygous and null cells, relative to wt (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.3.5.5 Expression of Hoxa1

Hoxa1 mRNA was significantly higher in null cells relative to wild- type cells (P<0.01). Expression in heterozygous cells was also slightly increased compared with wild-type cells (Figure 6-19).

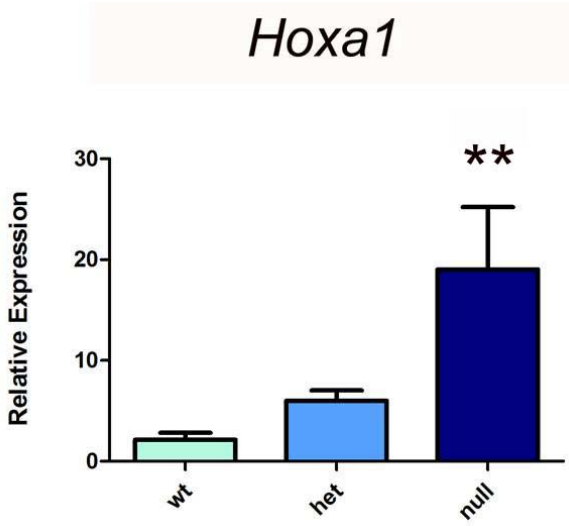


Figure 6-19 mRNA analysis for Hoxa1 in wild type, heterozygous and null cells, relative to wt (N=4). Taqman was performed in triplicate for 4 independent cultures for each cell line. Mean ± sem

6.4 Discussion

6.4.1 Expression of germ cell genes in undifferentiated ES cell lines

The three wild-type ES cell lines (EFC1, E14 and CGR8) examined all expressed high levels of *Oct4, Nanog* and *Sox2* consistent with previous reports that these genes are highly expressed in ES cells (Nichols et al., 1998; Chambers et al., 2003; Avilion et al., 2003). Sox2 and Oct4 have been shown to act together to drive the expression of a number of genes, the expression of which is associated with the maintenance of pluripotency (Okumura-Nakanishi et al., 2005), and they have been shown to heterodimerize and bind to the *Nanog* promoter (Rodda et al., 2005). Sox2, Oct4 and Nanog are also expressed in early mouse germ cells (Scholer et al., 1990; Yeom et al., 1996; Yamaguchi et al., 2005; Yabuta et al., 2006; Western et al., 2005; Perrett et al., 2008). Data from a recent study by Chambers et al. in 2007 in which chimeric mice were created from a mix of wild-type and *Nanog* -/- ES cell revealed that although the *Nanog* -/- cells were initially recruited to the germline, by e11.5 germ cells were lost.

In the current studies, the expression of both *Oct4* and *Nanog* mRNAs decreased significantly when cells were differentiated after five days of embryoid body formation, although expression was not completely lost, consistent with results reported in a previous study (Geijsen et al., 2004). After 5 days of differentiation, it has been proposed that the *Oct4* and *Nanog* positive cells may be the developing germ cells (Geijsen et al., 2004).

All three ES cell lines examined expressed *Fragilis*, *Blimp1* and *Stella* mRNAs at reasonably high levels compared with total tissue extracts from embryonic mouse testis. Other groups have reported expression of *Stella* and *Fragilis* in undifferentiated ES cells (Geijsen et al., 2004, Pain et al., 2005), and two studies have described the heterogeneous expression of *Stella* in ES cells (Payer et al., 2006; Carter et al., 2008). *Stella* expression has also been shown to be downregulated upon differentiation of human ES cells (Clark et al., 2004; Payer et al., 2003). The

expression of *Fragilis*, *Blimp1*, and *Stella* all decreased with five days of differentiation, suggesting that they are all genes associated with the pluripotent state. Expression of *Blimp1* has not previously been reported in undifferentiated ES cells but it is expressed in a large number of embryonic tissues (Vincent et al., 2005).

In this study all ES cell lines examined expressed *Mvh* in the undifferentiated state, although at far lower levels than the adult mouse testis. Interestingly, *Mvh* protein was easily detectable on Western blots prepared with extracts from all three ES cell lines. One group has also reported the mRNA and protein expression of *Mvh* in mouse ES cell (Kerkis et al., 2007), while others have found undifferentiated ES cells to be *Mvh* negative (Toyooka et al., 2003), although differing cell lines and culture conditions could explain the variability in expression. *Dazl* mRNA has also been reported in undifferentiated mouse ES cells (Geijsen et al., 2004; Kerkis et al., 2007) and Dazl protein has been immunolocalised to human ES cells (Clark et al., 2004).

Stra8 mRNA was expressed at low levels in the undifferentiated ES cell lines examined, and has previously been reported to be expressed in mouse ES cells (Kerkis et al., 2007), although expression did decrease after five days of differentiation. All three ES cell lines also expressed *Kit* mRNA, *Kitl* mRNA and *Hoxa1* at low levels. Previous studies have reported that the expression of *Hoxa1* increases as ES cells differentiate (Atkinson et al., 2008), and our results mirrored these findings.

Therefore the expression of genes associated with the germ cell lineage such as *Blimp1*, *Fragilis* and *Stella* has been demonstrated in ES cells. The expression of later germ cell markers *Mvh* and *Dazl* has also been shown in undifferentiated ES cells, despite their absence from the ICM. Additionally mRNAs for *Kit* and *Kitl* have both been shown to be expressed in ES cells.

6.4.2 *Kit* null cell express low levels of *Blimp1*, *Fragilis* and *Stella*

One hypothesis of this chapter was that Kit signalling may be important for the expression of germ cell genes in undifferentiated mouse ES cells. Microarray analysis revealed that *Kit* null cells, cultured in the presence of LIF, expressed several genes associated with the germ cell lineage at a lower level than the wild-type (Bashamboo and Forrester, unpublished). Results in this chapter confirmed that *Blimp1*, *Fragilis* and *Stella* mRNAs were all expressed at lower concentrations in the *Kit* null cell line compared with control E14s. Notably concentrations of *Oct4*, *Nanog* and *Sox2* mRNAs were indistinguishable between wild-type, heterozygous and null cells, in agreement with previous findings (Bashamboo et al., 2006). Heterozygous cells also displayed a significant reduction in *Blimp1* mRNA. However, the low levels of *Blimp1* expression in heterozygous cells appears not to be related to levels of *Kit* expression in the *Kit* heterozygous cells, as *Kit* mRNA is expressed in the heterozygous cells at comparable levels to the wild-type cells. This suggests that the significant reduction in *Blimp1* expression observed in the null cells may also not be related to the loss of *Kit*. This raises potential doubts about the role of Kit signalling on *Blimp1* expression.

The function of *Blimp1* is not fully understood, but it may have a role in epigenetic reprogramming of germ cells as it has been shown to interact with Prmt5, an arginine specific histone methyltransferase in mouse germ cells (Ancelin et al., 2006). *Blimp1* is thought to commit germ cells to the germ cell lineage by suppressing the expression of somatic genes, and inducing the upregulation of *Stella* and *Sox2* (Saitou et al., 2002; Yabuta et al., 2006; Kurimoto et al., 2008) (Chapter 1, 1.3.1.6).

The expression of *Hoxa1* was increased in the null cell line compared with the wild-type cells. This increased expression could be the result of loss of *Kit* or the reduction may be as a consequence of the reduced *Blimp1* expression observed in the null cells. Previous studies suggest that in germ cells, the Hox genes are downregulated as they become committed to the germ cell lineage and that Blimp 1 may be involved in this (Saitou et al., 2002; Ohinata et al., 2005). However, in the

heterozygous cells, although *Blimp1* is significantly downregulated, *Hoxa1* expression is not upregulated, weakening the argument for a relationship between *Blimp1* and *Hoxa1* expression.

Ideally further analysis has to be performed to determine if any other lineages are affected in the Kit null cells, other than just the germ cell lineage. Although the array analysis of early cardiomyocyte, neuronal and adipocyte markers doesn't suggest that there are any changes in these lineages, a more comprehensive analysis would be useful, as Kit signalling may simply act by controlling the aberrant expression of a number of transcripts in ES cells.

Immunomagnetic bead sorting of the wild-type E14 and EFC1 cell lines using an anti-Kit antibody, resulted in a significant reduction *Kit* expression in the cells bound to the beads than those that were bound. This in agreement with the finding of others, that undifferentiated ES cells express *Kit* in a heterogenous manner (Lu et al., 2007). RNA analysis of these two cell populations in both cell lines showed that expression of *Oct4*, *Nanog* and *Sox2* did not vary between the *Kit* 'low' and *Kit* 'high' populations. However in a previous FACS sorting study by Lu et al. (2007), the *Kit* low/negative population have been shown to express significantly lower levels of *Nanog*. In this study the expression of *Blimp1* mRNA was significantly lower in the *Kit* 'low' population for both the E14 and EFC1 cells.

6.4.3 ES cells may contain a germ cell niche

Further study of the spatial distribution of germ cell gene expression in undifferentiated ES cells is required to understand the regulation of germ cell gene expression in ES cells. Payer et al (2006) used Stella-GFP reporter ES cells and found that the *Stella* positive cells occurred in distinct subpopulations, possibly indicating that within an ES cell colony there are germ cell-like ES cells (Aflatoonian and Moore, 2006; Payer et al., 2006). We might speculate that Kit/Kitl signalling is important for maintaining these germ cell-like subpopulations of ES cells and this

would be consistent with the presence of the *Kit* positive and *Kit* negative cells in our cultures.

As germ cells develop within a differentiating ES cell culture system, we assume that a niche is created, which resembles the normal *in vivo* microenvironment. Studies have shown that the formation of male germ cells requires embryoid body differentiation and the use of a 2D culture system only results in the formation of female germ cells (Hubner et al., 2003; Kerkis et al., 2007). It has been reported that within the differentiating embryoid body, male germ cells tend to form on the surface, while female germ cells form inside the embryoid body (Kerkis et al., 2007). Directed differentiation of male germ cells has also been reported with successful derivation of developing male germ cell occurring when ES cells are allowed to differentiate in the presence of both retinoic acid (RA) and testosterone (Silva et al., 2008) or testicular cell-conditioned media containing cytokines such as BMP4 and GDF-9 (Lacham-Kaplan et al., 2006). If it is the case that a 'germ cell- like' phenotype is enhanced by the Kit/Kitl signalling, one possible way of enhancing germ cell formation during differentiation is to supplement the media with Kitl. However, the addition of Kitl to ES cell cultures has been shown to have no effect (Lu et al., 2007) possibly because the ES cells are themselves are already producing Kitl (Lu et al., 2007).

6.4.4 Conclusion

The expression of several germ cell- associated genes in different ES cell lines has been analysed. Wild-type cells express a variety of germ cell markers in the undifferentiated state, raising questions about whether there are cells within an undifferentiated ES cell colony that have begun differentiating down the germ cell lineage or whether ES cells express germ cell genes as a consequence of their pluripotency. Some of these genes (*Blimp1, Fragilis* and *Stella*) are expressed fairly highly in the undifferentiated state, and decrease upon differentiation. Other germ cell specific genes such as *Mvh* and *Dazl* increase upon differentiation, suggesting the germ cell-like cells could be forming after 5 days of embryoid body

differentiation. Analysis of the immunomagnetically bead sorted population, with low levels of *Kit* gene expression, have shown that the cells exist as a mix of *Kit*+ and *Kit*- cells and that the expression of *Blimp1* is downregulated in the absence of *Kit*. In the *Kit* null cells, *Blimp1*, *Fragilis* and *Stella* are expressed at lower levels. This would suggest that Kit dependent signalling may have some degree of influence on the expression of these genes in ES cells.

Although it cannot be directly inferred that these *Kit* null ES cells have a less germ cell-like phenotype it could be speculated that the *Kit* null cells ES cells may be less capable of form ES cell derived germ cells. However, there are several questions which this study has not addressed, for example, the reduction in *Blimp1* expression in the heterozygous cells may suggest that in the null cells the reduced expression of Blimp1 may not be the direct result of a loss of Kit signalling, and further studies on new *Kit* heterozygous and null clones are required. Additionally, with the exception of Mvh, no protein expression for any of these germ cell genes was successfully detected within the undifferentiated ES cells. One possibility is that within ES cells there are a number of abundant transcripts which are not translated into protein. Kit signalling may control the trancription of a number of these transcripts, and a more comprehensive analysis would be useful as Kit signalling may simply act by controlling the aberrant expression of a number of transcripts in ES cells.

7 Final discussion

Germ cells are responsible for the transmission of genetic material between generations. During fetal life germ cells must be established from pluripotent precursor cells, migrate into the developing gonad, where they can downregulate pluripotency genes and develop down either the spermatogonial or oogenic lineage. In the female, germ cells in the fetal ovary stop dividing and enter meiotic prophase I, only to arrest at the diplotene stage (McLaren and Southee, 1997). In the developing male gonad, germ cells undergo differentiation and mitotic arrest, but do not enter meiosis until puberty. The normal development of germ cells is crucial for future fertility, and, the abnormal differentiation of germ cells can result in malignant germ cell tumours (Skakkebaek, 1972; Rajpert-De Meyts, 2006). The activation of sex-specific genes in the somatic cells of the gonad is critical in determining the pattern of maturation of germ cells in the two sexes. The majority of research into germ cell development during fetal life, and especially those studies aimed at understanding the role the somatic cells, has utilised rodent models. However, studies which have focussed on germ cell development in the human fetus have shown that there are several key differences from the process occurring in rodents (Gaskell et al., 2004; Honecker et al., 2004). The main objective of the work described in this thesis was to obtain a better understanding of human fetal germ cell development and the impact which the somatic cells have, primarily in the human fetal gonad. In addition, data suggests that further insight into germ cell establishment and differentiation can be obtained by studies using embryonic stem (ES) cells (Toyooka et al., 2003; Geijsen et al., 2004; Hubner et al., 2003), therefore, a study exploring germ cell gene expression in mouse ES cells was conducted.

The specific aims of the studies described were: 1) to further characterize the subpopulations of germ cells in the human fetal testis, by documenting protein expression patterns through the use of double fluorescent immunohistochemistry; 2) to determine whether the mechanisms leading to retinoic acid based regulation of meiotic initiation in the mouse fetal ovary and meiotic inhibition in the mouse fetal

testis, are conserved in the human fetal gonads; 3) To establish cultures of human fetal testicular somatic cells in order to further understand gene expression in the 2nd trimester human fetal testis; 4) To study the expression of germ cell genes in mouse ES cells and determine the role of the Kit receptor in the germ cell gene expression.

7.1 Characterisation of germ cells in the human fetal testis

Previous studies into germ cells in the human fetal testis have shown that distinct germ cell subpopulations can be identified based on their morphology (Wartenberg, 1981) and their protein expression pattern. Notably the 'less mature' OCT4 positive gonocytes and 'more mature' OCT4 negative prespermatogonia, can be found residing within one seminiferous cord during the 2nd trimester (Gaskell et al., 2004; Honecker et al., 2004).

This current study has expanded on our previous knowledge of the protein expression profile of germ cells in the human fetal testis. Through the use of double fluorescent immunohistochemistry, the OCT4-positive (gonocyte) population have been shown to express M2A, NANOG, AP2γ and DAZL. The OCT4-negative prespermatogonia have been shown to express low/negative levels of M2A and AP2γ, while expressing some DAZL and high levels of VASA and NANOS1 (summarised in Figure 7-1). The confined expression of VASA to the prespermatogonial population was confirmed by Western blot, which revealed that VASA protein could not be detected in extracts from 1st trimester testis. In the 2nd trimester testes, a putative 'intermediate' germ cell population has previously been identified, these are cells that were thought to be undergoing the transition from a gonocytes phenotype into the prespermatogonia (Gaskell et al., 2004). In the current study, germ cells were identified that had reduced immunopositive staining for OCT4 compared with adjacent gonocytes, and also had a low level of expression of VASA, a marker considered characteristic of the spermatogonial population. Although no single protein exclusively localised to the intermediate cell population was found, it was notable that these cells were also marked by a relocation of DAZL

from the nucleus to the cytoplasm. These observations provide more evidence for the gradual differentiation which occurs in human fetal testicular germ cells.

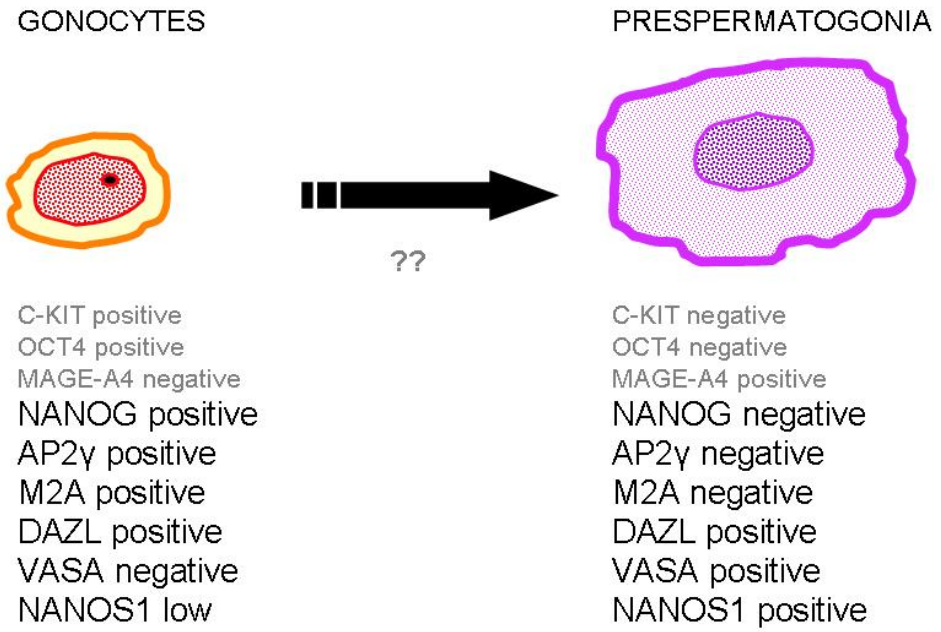


Figure 7-1 Summary of main findings regarding protein expression in human fetal gonocytes and prespermatogonia. Grey text refers to data reported by Gakell et al, 2004, black text refers to novel findings reported in this thesis.

Strikingly, analysis of testicular germ cell tumour samples revealed that within tubules containing carcinoma in situ (CIS), the OCT4-positive CIS cells expressed low/negative levels of VASA. Previous studies have described VASA as a CIS cell marker (Honecker et al., 2004; Zeeman et al., 2002), but its expression in relation to other proteins known to be expressed in CIS had not been investigated. The findings in the current study therefore raise questions as to whether some CIS cells do express VASA or whether the VASA positive cells are 'normal' CIS cells confined to a tubule which contains OCT4 positive CIS cells. If the OCT4 positive/VASA negative cells are the true CIS population, then this would suggest that CIS cells arise

from the gonocytes population, alternatively, the VASA positive/OCT4 negative cells may also be CIS cells that have undergone some degree of differentiation. Further analysis on a larger number of samples needs to be performed and it would be interesting to determine whether other mature markers, such as NANOS1, are also distinct from the OCT4 positive population. Germ cell maturation takes place gradually over weeks, during this time, any adverse changes in the cellular environment might contribute to a failure of normal differentiation (i.e. predispose to CIS), and this may be the reason why humans have CIS and rodents do not.

Previous reports have demonstrated that germ cells within the human fetal gonad proliferate throughout the 1st and 2nd trimester (Honecker et al., 2004; Murray et al., 2000). This study has confirmed and extended these finding by showing that Ki67 positive germ cells are present throughout the 2nd trimester, and although towards the end of the 2nd trimester, proliferation appeared to decrease, this was not statistically significant. It would have been interesting to study germ cell proliferation beyond the 19 week period examined as Honecker et al. in 2004 reported that germ cell proliferation persisted throughout the 3rd trimester. Notably the current study has revealed that germ cell proliferation was not restricted to either of the two main germ cell populations, as both the gonocytes and prespermatogonia were immunopositive for Ki67. Fluorescent immunostaining of 1st trimester testis revealed a large number of Ki67 positive germ cells, ideally it would have been useful to quantify the numbers of proliferating germ cells throughout the 1st trimester, however accurate identification of germ cells within the 1st trimester gonad proved technically challenging and subsequent attempts to use non-fluorescent dual staining for identification and counting were unsuccessful.

This study has improved upon knowledge of protein expression patterns in germ cells of the human fetal testis and moreover, it has highlighted the similarities in germ cell maturation which occur between fetal male and female gonads (Anderson et al., 2007). Both germ cells in the ovary, as well as the testis, show a similar overlapping pattern of expression despite some germ cells in the ovary entering

meiosis (Anderson et al., 2007). In the ovary, OCT4 positive germ cells were confined to the peripheral cortex, while VASA positive cells were low/negative for OCT4 and were found in larger cells located towards the centre of the ovary (Anderson et al., 2007). This study has also contributed to a parallel study on the common marmoset (Callithrix jacchus) (Mitchell et al., 2008). The protein expression pattern found in the human was similar in the marmoset, and, as both species appear to display a similar slow germ cell differentiation, the marmoset may provide a good model for studying germ cell development, and this could be a future model of CIS (Mitchell et al., 2008).

7.2 Factors regulating the control of meiotic entry in the human fetal ovary and testis

Studies suggest that in the mouse, retinoic acid (RA) plays a key role in controlling meiotic entry. In the developing mouse, RA appears to be released from the mesonephros, and in the female, possibly through direct signalling to the germ cells (Koubova et al., 2006; Bowles et al., 2006), RA appears to initiate meiosis, perhaps by upregulating *Stra8*. In the mouse testis, although RA is also synthesised in the mesonephros, the expression of the enzyme *Cyp26b1* in the testicular somatic cells ensures degradation of RA and blocks the signal for meiosis (Koubova et al., 2006; Bowles et al., 2006). The present study sought to determine if RA activation was also likely to operate in the human fetal gonad by examining the expression of enzymes and receptors involved in RA biosynthesis and action.

To date, there have been no published reports documenting *STRA8* expression in the human fetal gonads. This study has shown that total concentrations of *STRA8* mRNA were significantly higher in extracts of ovary than testis between 14-15 weeks gestation. Notably this is the time when female germ cells are entering meiosis, suggesting that the functions of STRA8 may also play a critical role in human gametes as has been suggested by studies in the mouse (Menke et al., 2003; Baltus et al., 2006; Koubova et al., 2006). These studies would have been enhanced

if the specific cells within the human fetal ovary expressing *STRA8* could have been identified either using in-situ hybridisation or immunohistochemistry, in order to determine whether *STRA8* was restricted to the premeiotic germ cells, as has been reported in the mouse. However, no specific antibody was available and further optimisation is needed of in-situ hybridisation methodology. Additionally, analysis of cell-specific expression of meiotic markers such as SYCP3 could provide an additional marker when studying the spatial distribution of germ cells in the human fetal ovary as they enter meiosis, since current information is based only on size and gross appearance. Recent studies in the mouse suggest that Nanos2 may play a role in the suppression of *Stra8* in the male (Suzuki and Saga, 2008) and our novel finding that expression of *NANOS2* mRNA was restricted to the human fetal testis would be consistent with this idea, but again a limitation with this study is the fact that the identity of the cells within the fetal testis expressing *NANOS2* was not determined and therefore further validation is required on testes sections.

In support of the hypothesis that RA may have an impact on germ cell maturation in the human as well as the mouse, all RARs and RXRs were found to be present during both the 1st and 2nd trimester in both the human fetal ovary and testis. Furthermore, protein expression of RARα, RARβ and RXRα were demonstrated in both the germ cells and somatic cells in both sexes. This is in agreement with studies in the rodent where germ cells and somatic cells of the testis and ovary are immunopositive for many of the RA receptors (Boulogne et al., 1999; Morita and Tilly, 1999). To our knowledge this is the first study examining RA receptor expression in the human fetal gonad. Interestingly, the receptors RARα, RARβ and RXRα were immunolocalised to the cytoplasmic compartment of a number of cell types, as well as the nuclei of other cells. Cytoplasmic staining was particularly prevalent in female germ cells, suggesting that they may be awaiting targets for stimulation by RA (Morita and Tilly, 1999).

In the mouse, the mesonephros has been suggested as the likely site of RA biosynthesis (Bowles et al., 2006). In this study although the human mesonephros

was found to contain the mRNAs for two enzymes involved in RA biosynthesis, the concentrations appeared to be lower than the amounts in gonadal extracts. However since the retinaldehyde dehydrogenase enzymes are only a small part of a larger biochemical pathway leading to the production of RA, simply comparing the levels of transcript between tissues cannot be considered as a comprehensive method for determining the amount of RA produced by the cells. Therefore a reporter cell line was employed to assess whether RA was released by the mesonephroi or produced by the gonads. Disappointingly the initial studies have failed to yield conclusive results and questions exist about the sensitivity of the cell line. Another potential method for measuring the levels of RA within tissues is through the use of high performance liquid chromatography (HPLC) (Kane et al., 2008). Future studies will explore whether this method would allow a complete chemical analysis of the levels of RA in the developing ovaries, testes and mesonephroi.

In the mouse *Cyp26b1* is expressed at high levels in the testis at e13.5, at the time when germ cells in the ovary are entering meiosis (Koubova et al., 2006; Bowles et al., 2006). In the present study, analysis of the concentrations of *CYP26B1* mRNAs found in the developing human gonad actually suggests that total amounts are higher in the ovary than the testis. In the mouse *Cyp26b1* mRNA has been localised to the Sertoli cells and PTM cells of the testis using in-situ hybridisation (Koubova et al., 2006; Bowles et al., 2006). In our study we have as yet not been able to localise the expression of *CYP26B1* mRNA or protein to specific cell types in the human fetal gonads. A full understanding of the role of CYP26B1 will require that we ascertain the cell types within the developing ovary and testis that express *CYP26B1*. Additionally, no studies in the mouse have reported localisation of Cyp26b1 protein and studies into the protein expression patterns of CYP26B1 in both the mouse and human are vital in order to demonstrate any potential function.

A number of groups have reported effects of RA on germ cells *in vitro* (Koubova et al., 2006; Bowles et al., 2006; Koshimizu et al., 1995; Morita and Tilly, 1999). For example, RA treatment is reported to induce a decrease in expression of *Oct4* and an

upregulation of *Stra8* in intact gonads and upon incubation with a RA antagonist, the opposite occurs (Koubova et al., 2006). In order to obtain a better understanding of the role of RA within the human fetal gonad, it would be useful to perform treatments on gonadal cells or explants *in vitro*. For example, it would be interesting to determine whether RA has any direct effect on gene expression in germ cells in the human fetal ovaries and testes, and what effect treatment with the cytochrome inhibitor ketoconazole or a RA receptor antagonist has on germ cell development.

The total amount of *SDMG1* mRNA, was higher in the testes than in the ovaries, particularly towards the later part of the second trimester. The transmembrane protein SDMG1 is believed to be part of a large network of proteins which control the release of peptides (Best et al., 2008). It has been proposed that Sdmg1 plays a vital role in the control of molecular release from the Sertoli cells in the mouse, at the time when germ cells are committed to the male lineage, preventing meiotic entry. In our study, expression of, *SDMG1* appeared to be upregulated in the testes late into the second trimester (18-19 weeks). The time when germ cells commit to the male lineage is unknown, but is estimated to be between 8-11 weeks gestation. A comprehensive analysis of protein expression and cellular localisation must be performed to give further insight into SDMG1 function in the human fetal testis. Further analysis of proteins associated with membrane trafficking would be of interest, to determine whether these genes are upregulated in the Sertoli cells of the human fetal testis.

7.3 Establishment of cultures of human fetal testicular somatic cell

From the studies described above, and also other data, including that on the functional maturation of Sertoli cells (Brennan and Capel, 2004), the somatic cells of the testis play a key role in modulating germ cell fate (Palmer and Burgoyne, 1991). Studies analysing gene expression in the somatic cells of the human fetal testis are limited. One potential method for studying the somatic cells is through *in vitro* culture, but as yet there have been no reports in the literature of the successful *in vitro* culture of somatic cells from the human fetal testis.

The principle aim of this part of the study was to establish a culture system for testicular somatic cells so as to provide a tool allowing for the characterisation and modulation of gene expression. Analysis of the levels of expression of mRNAs of Sertoli cell specific transcription factors, *SRY* and *SOX9,* revealed that these were detectable in cells from all 5 passages of cultured cells, although the expression of both decreased with increasing passage number. However, expression of other Sertoli cell genes, *AMH* and *DHH* are almost immediately downregulated. These data suggest that the Sertoli cells are surviving in the cultures but with an altered phenotype, and may have reverted back to a more primitive state, expressing only the earliest markers of Sertoli cell differentiation. Despite the loss of *AMH* expression, a number of genes implicated in the control of AMH transcription (reviewed by Lasala et al., 2004) were found to be present within the cultures, including *SRY* and *SOX9* and also *SF1* and *GATA4*, and this warrants further investigation. Also detectable within the cultures were mRNAs for the Leydig cell markers, *3BHSD* and *SCCp450*, however, expression of these genes was almost completely lost by passage 2, indicating an early loss of differentiated/functional Leydig cells.

In contrast to the decrease in gene expression observed for the Sertoli cell markers and the Leydig cells markers, total expression of mRNAs normally detected in PTM/Fibroblasts appeared to increase in cell extracts. This suggested that within the cultures that were initially established as a mixed population of somatic cells, overgrowth by fibroblasts was occurring. Interestingly, AR was expressed both within the cultures and in fixed human fetal testis sections, where it was immunolocalised to both the fibroblast and PTM populations within the interstitium. Although we predict that these were the two cell populations that were increasing as a proportion of the total cell population in the cultures, *AR* mRNA concentrations decreased. This suggested to us that the PTM/fibroblast population were also dedifferentiating within the cultures, despite the stimulation by DHT. DHT was supplemented to the media to mimic the *in vivo* environment and in order to maintain the PTM population. However in doing so, DHT may have positively selected for the fibroblast population, aiding their overgrowth. With hindsight, an initial

experiment should have been performed comparing gene expression following treatment with DHT with untreated cultures, rather than supplementing the media with DHT in all cases.

Passaging the cells with dispase rather than trypsin resulted in a slight improvement to the survival/phenotype of Leydig cells and Sertoli cells, but for only a short period of time. The cells in the cultures were found to express the activin receptor subtypes, and this would be consistent with reports that a variety of cells within the human fetal testis express all of the required components for activin signalling and production (Majdic et al., 1997; Anderson et al., 2002). However treatment with activin A caused no alteration in gene expression in the cultured cells. These negative results may be because activin was incapable of signalling within the cultured cells or because activin induced no effects on the expression of genes analysed (*AMH, DHH, SOX9* and *SMA*). Further experiments are required to investigate the potential for activin signalling by analysing whether the cells expressed the protein of the activin receptors and whether treatment with activin could induce any phosphorylation of SMAD proteins, but due to limited tissue and time, this was not performed.

A more comprehensive analysis of protein expression within these cells would also be beneficial. SOX9 and GATA4 were both immunolocalised to subpopulations of the cells, however it was never determined whether these two proteins were expressed within the same cell type. By performing dual staining to analyse this, more information would be obtained on the gene expression in the Sertoli cells, since GATA4 was also expressed in the PTM/fibroblast population.

A common problem with primary tissue culture is the overgrowth by fibroblast (Singer et al., 1989). The culture system could perhaps be improved if methods for removing or reducing fibroblasts are performed. Fibroblast growth is enhanced in serum-containing media, so a strategy of optimisation of the culture of these cells in serum-free media may have proved useful at reducing fibroblast numbers. There are

several reported methods for elimination of fibroblasts such as selective trypsinization (Harris, 1996) or their isolation using cell sorting procedures (Saalbach et al., 1997), and future experiments could attempt these methods.

Further work must therefore be performed to try and maintain the differentiated phenotype of the cells. Improved culture of somatic cells may also be achieved through culturing the cells on a basement membrane, for example with laminin or matrigel. There are reports of reasonably successful culture of rodent Sertoli cells when they are cultured on an extra-cellular matrix (ECM) (Gassei et al., 2006). Others have made progress in culturing mouse ovarian tissues and neonatal testis in a 3D cultures system with a ECM such as matrigel or sodium alginate (Xu et al., 2006; Hadley et al., 1985; Noguchi et al., 2006; Gassei et al., 2006). It would be interesting to see if when subjected to this sort of environment, if human fetal testicular somatic cells maintained a normal phenotype for longer. The ultimate aim with this study would be to generate a culture system where somatic cells are remixed with germ cells in order to support their development and/or determine those Sertoli cell or PTM cell factors critical for germ cell maturation.

7.4 Expression of germ cell genes in mouse ES cells and the role of *Kit*

One of the principle aims of this part of the project was to characterise the expression of genes normally considered to be germ cell specific in mouse ES cells, prompted by several reports claiming successful establishment of germ cells from ES cells (Geijsen et al., 2004; Pain et al., 2005; Kerkis et al., 2007; Hubner et al., 2003); although it is notable that several groups have also reported the expression of early germ cell marker in undifferentiated ES cell cultures (Toyooka, et al, 2003; Geijsen et al., 2004; Reviewed by Zwaka et al, 2005). The mRNAs for genes such as *Fragilis, Stella* and *Blimp1* was consistently detected in all three cell lines. Although others have reported the expression of *Stella* and *Fragilis* in ES cells (Geijsen et al., 2004; Pain et al., 2005), to our knowledge this is the first report of *Blimp1*

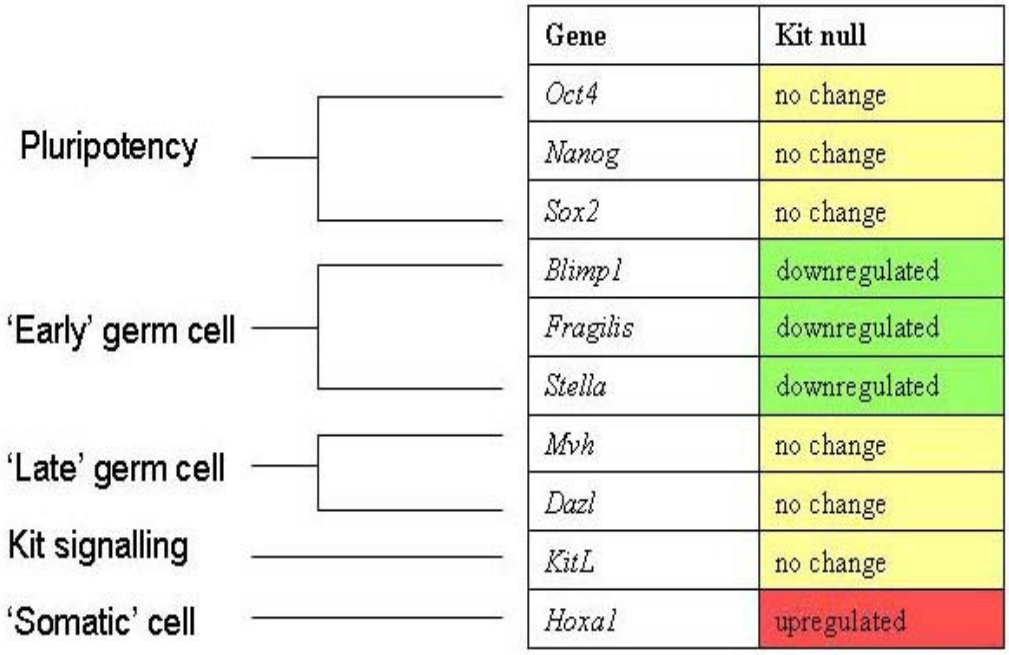
expression. Upon embryoid body differentiation, expression of these genes decreased, along with the pluripotent genes, implicating *Fragilis, Stella* and *Blimp1* with the pluripotent state. The expression of slightly later germ cell markers, *Mvh* and *Dazl* was also demonstrated in all ES cells, although at much lower levels than adult mouse testis. One limitation of this part of the study was that mRNA was analysed from whole ES cell cultures. It would be exciting to look at the spatial expression of genes such as Blimp1 and Stella throughout the cultures, and determine through the use of immunohistochemistry, whether distinct subpopulations within the cultures express these genes at the protein level. For example it would be interesting to investigate whether the Blimp1 positive cells within the cultures also expressed Stella and Fragilis.

The expression of both *Kit* and *Kitl* mRNAs has confirmed previous studies which have detected both of these mRNAs in ES cell cultures (Bashamboo et al., 2006; Lu et al., 2007). Previous studies suggest that Kit/Kitl signalling plays an important role in modulating gene expression in ES cells during the early stages of differentiation. In ES cells null for the *Kit* receptor, microarray analysis pointed to several genes important for the early stages of germ cell differentiation to be downregulated, namely *Blimp1*, *Fragilis* and *Stella* (Bashamboo and Forrester, unpublished). To determine whether sub-populations of ES cells enriched or lacking expression for the Kit receptor could be obtained, immunomagnetic bead sorting using an antibody directed against Kit was performed. Cells bound to the immunomagnetic beads displayed a significantly higher expression of the *Kit* mRNA compared to those that were unbound confirming that the cell-sorting had been successful. In a comparison of the levels of expression of pluripotency genes between the bound and unbound population of cells, levels of *Oct4, Nanog* and *Sox2* were found to be similar, and it was concluded that Kit signalling was not required to maintain the pluripotent state of ES cells. This is in agreement with the findings of others (Bashamboo et al., 2006; Lu et al., 2007). Analysis of the *Kit* 'high' and 'low' populations may have been improved if fluorescent activated cell sorting (FACS) had been performed instead of the immunomagnetic beads. FACS may have allowed for a purer

population of cells to be obtained and flow cytometry would also have been a useful way of quantifying the number of cells expressing Kit in these cell cultures. In the *Kit* 'high' population of cells, *Blimp1* was found to be significantly higher than in the *Kit* 'low' cell population. The expression of *Fragilis* and *Stella* was also found to be reduced slightly in the *Kit* 'low' population. These results were enhanced by analysis of a *Kit* null ES cell line (summarised in Table 7-1). The expression of *Blimp1*, *Fragilis* and *Stella* were all reduced significantly in the null cells compared with the wild-type. Heterozygous cell also showed decreased expression of these genes. More mature germ cell markers such as *Mvh* and *Dazl* appeared to be largely unaffected by the lack of *Kit*. Interestingly, despite the absence of *Kit* in the null ES cells, *Kit* ligand expression was normal. This shows that in the ES cells, Kit ligand expression is not under the control of its receptor.

One key prerequisite for the successful establishment of the germ cell lineage *in vivo*, appears to be the suppression of the somatic cell program, and cells destined to become germ cells, downregulate genes associated with the somatic cell lineage (Saitou et al., 2002; Ohinata et al., 2005). In this study *Hoxa1* was found to be higher in the *Kit* null and *Kit* 'low' cells, which could perhaps support the hypothesis that they are less germ cell-like, however, analysis of other genes such as *Hoxb1*, also downregulated in cells at the very earliest stages of germ cell commitment is also necessary.

Table 7-1 Summary of germ cell gene expression in Kit null ES cell lines



Gene	Kit null
Oct4	no change
Nanog	no change
Sox2	no change
Blimp1	downregulated
Fragilis	downregulated
Stella	downregulated
Mvh	no change
Dazl	no change
KitL	no change
Hoxa1	upregulated

In addition to the null cell line, Bashamboo et al., 2006 also generated an inducible cell line, where the ES cells carried a null allele of Kit W^{lacz} and a knock-in allele $W^{FKB\text{-}kit}$, encoding a Kit ligand-independent hybrid receptor that becomes activated upon stimulation by the drug AP20187, which acts by binds the FKB receptor. Treatment with this drug has previously been shown to rescue the *Kit* null cell phenotype (Bashamboo et al., 2006). Studies using the inducible cell line would be useful to verify the findings of the current study on the *Kit* null cells and critically whether the drug would cause the cells to upregulate their expression of *Blimp1, Fragilis* and *Stella*, confirming them as down-stream of Kit functional activation. It will be necessary to repeat the RT-PCR analysis on this inducible line to ensure that the altered phenotype of the null cells we studied is not as a result of clonal effects, however in a set of array data, the cell line containing the knock-in allele $W^{FKB\text{-}kit}$, also display reduced expression of *Blimp1, Fragilis* and *Stella* (Bashamboo and Forrester, unpublished).

In order to fully understand the effect of Kit signalling on germ cell formation from ES cells, ES cell-derived germ cells need to be created from the mutant ES cells.

However, this would be difficult given that the *Kit* null cells die by apoptosis following LIF withdrawal (Bashamboo et al., 2006). In order to study germ cell formation in the differentiating *Kit* null cells, embryoid bodies would need to be allowed to form in the presence of LIF, allowing mutant cells to survive the early stages of differentiation due to the presence of LIF, using the system previously described by Geijsen et al., 2004. The hypothesis of the involvement of Kit/Kitl signalling controlling germ cells formation from ES cells is based solely from the data presented in this thesis, and that there is no *in vivo* data pointing to a role of Kit/Kitl in germ cell formation, as germ cells do initially form in embryos deficient in *Kit* and *Kitl*.

ES cells consist of subpopulations of cells that exhibit distinct gene expression profiles (Hayashi et al, 2008). Kit signalling is unlikely to be important for maintaining pluripotent stem cells within the ES cell culture, as Nanog, Sox2 and Oct4 expression is normal in the Kit null cells and the Kit low cells. Kit signalling however may be important for the maintainance of differentiated cells present within the cultures. Germ cell gene expression within the ES cell cultures may reflect distinct subpopulations of cells, rather than the homogenous expression of germ cells markers in ES cell cultures. An example of this is Stella, which has been shown to be expressed in a subset of ES cells (Hayashi et al, 2008; Payer et al, 2006).

7.5 General Conclusions

In conclusion, results obtained during the current study have enhanced our knowledge of fetal germ cell development and differentiation, and the potential impact of the somatic cells. Further characterisation of protein expression patterns in germ cells of the human fetal testis has been performed and proteins have been shown to localise exclusively to the gonocytes population, while other proteins have been shown to be restricted to the prespermatogonia. This study also raises questions about whether the proposed model controlling the meiotic entry of germ cells in the mouse can be applied to the human, several genes known to be expressed in a sex-

specific manner in the mouse, have shown a similar pattern in the human, but other genes involved in RA production and metabolism appear to show a different expression pattern in the human. The *in vitro* culture of human fetal somatic cells may lead to further understanding of gene expression in the human fetal testis and could potentially lead to studies on germ cell-somatic cell interaction, while data suggests that the Kit/Kitl signalling system may control the expression of early germ cell genes in mouse ES cells.

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